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Transcriptional level of genes involved in the neurotransmitter system of *Dicentrarchus labrax* in response to chronic exposure to psychopharmaceuticals

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Abstract

In about 10% of citizens of European Union (EU) take antidepressants. In Portugal, according to OCDE, the use of antidepressants is higher than EU average. During the last decades, a variety of antidepressants, selective serotonin reuptake inhibitor (SSRI), as fluoxetine (FLUX), and serotonin norepinephrine reuptake inhibitor (SNRI), as venlafaxine (VEN), were recognized as an important source of environmental contamination and increasing concern emerged regarding their potential ecological impact. Pharmaceutical targets are highly conserved among vertebrates suggesting that aquatic animals, as fish species, are likely to be affected by environmental exposure even at low concentration (ng/L). Main therapeutic properties of these psychotropic drugs are based upon modulation of important monoamine neurotransmitters, as serotonin and dopamine. They block monoamine reuptake in the presynaptic cells, which leads to an increase in the concentration of serotonin and/or dopamine and norepinephrine within the synapses. Recent studies have focused on effects in freshwater fish, but information on marine fish is scarce. However, knowledge of the effects of PP on the neurotransmitter and neuroendocrine system is needed, which is important to evaluate risks for aquatic vertebrates, and finally on environmental and human health.

In this work, we explored and evaluated the transcriptional levels of neurotransmitters and receptors mRNA in the brain of the *Dicentrarchus labrax* in response to chronic PP exposure. Juvenile European Sea bass was exposed to two selected antidepressants, (i) FLUX at the concentration of 0.5 µg/L and 50 µg/L, and (ii) VEN at the concentration of 0.01 µg/L and 1 µg/L. The chronic exposures were performed for 21 days, followed by a 7 day depuration period, to assess the reversibility of effects. Transcriptional levels of genes encoding for proteins involved in the neurotransmitter system, such as *5-ht*$_{3a}$ and *5-ht*$_{3b}$, *sert, mao, vmat* and dopamine d_2 and d_3, were analyzed using qPCR-RT.

Different profiles of mRNA expression levels were obtained after exposure to SSRI and SNRI. Exposure to FLUX resulted in an acute response at day 1 in all analyzed target genes, except for the monoamine degrading enzyme, *mao*, which was increased after 21 days. Exposure to VEN caused a significant differences at day 21 for the receptor of serotonin *5-ht*$_{3a}$. For both serotonin receptors lower mRNA level were detected, but only for *5-ht*$_{3a}$ with significant different. Dopamine d_2 and d_3 receptors were significantly increased at 7 day of the recovery period.

These are important changes in the neuronal system of a marine fish species. The results suggest that antidepressants are able to modulate the expression of important neurotransmitter genes and receptors. We verified that genes from the analyzed genes in Sea bass had high degree of homology with *D.rerio*, *O.latipes, T.rubripes*, including *H. sapiens* and that all sequences clustered well within each corresponding group in a phylogenetic tree based on the multiple alignment analysis. Thus, we can assume that genes in *D.labrax* may have similar functions as in vertebrates. In humans, alterations of neurotransmitter genes and receptors are associated to human disorders as Alzheimer or Parkinson. For aquatic vertebrates, important functions include the regulation of anxiety, food intake, and aggression amongst others.

The combined analysis of two classes of antidepressants on a marine fish species revealed substantial differences in the physiological response of neurotransmitter genes and receptor mRNA expression. Results should be considered in future risk assessments of pharmaceuticals disrupting the neuroendocrine system, which highlight the need of an individual evaluation of environmental compounds, even if similarities of effects could be assumed.

Resumo

A depressão tem vindo a aumentar gradualmente ao longo destas últimas décadas, sendo que 1 em cada 10 indivíduos da União Europeia são afetados por esta doença. Os fármacos atualmente com maior número de prescrições médicas para o tratamento da depressão são o Prozac (fluoxetina), um potente inibidor seletivo da recaptação da serotonina (SSRI) e o Effexor (venlafaxina), inibidor da recaptação da serotonina e da norepinefrina (SNRI). As pesquisas mais recentes focam-se na preocupação destes fármacos serem reconhecidos como uma importante fonte de contaminação ambiental revelando que os alvos dos antidepressivos são conservados em termos evolutivos logo podendo impactar outros vertebrados. Como os neurotransmissores regulam uma grande variedade de sistemas fisiológicos desde mamíferos, peixes, moluscos e protozoários, espera-se que a presença dos psicofármacos possam afetar estes organismos a vários níveis, pois as principais propriedades terapêuticas destes fármacos são baseadas na modulação dos neurotransmissores.

Neste trabalho avaliamos a transcrição de mRNA de genes do sistema serotonérgico e dopaminergico no cerebro do *Dicentrarchus labrax* através da técnica PCR em tempo real. Selecionou-se do sistema serotonérgico os genes: *sert*, *5-ht*$_{3A}$, *5-ht*$_{3B}$; do sistema dopaminérgico: *d*$_2$, *d*$_3$; transportador não específico das monoaminas: *vmat* e por último uma enzima com atividade catalítica: *mao*. O *Dicentrarchus labrax* foi exposto a dois antidepressivos, (i) a fluoxetina com concentrações de exposição de 0,5 µg/L e 50 µg/L, e (ii) a venlafaxina com concentrações de exposição de 0,01 µg/L e 1 µg/L. As exposições crónicas foram realizados durante 21 dias, com um período de recuperação de 7 dias para avaliar a reversibilidade dos efeitos. Diferentes perfis de transcrição foram observados após a exposição a antidepressivos da classe SNRI e SSRI, (i) aumento da transcripção de todos os genes após exposição à fluoxetina (50 µg/L) no primeiro dia demonstrando um efeito agudo, exceto a enzima degradadora, a *mao*. Esta apresentou não só um aumento agudo na expressão, mas também apresenta um efeito crónico na concentração mais elevada (50 µg/L); (ii) na exposição à venlafaxina o gene do sistema serotonérgico, *5-ht*$_{3A}$, mostrou diferenças significativas no dia 21 com indução nos níveis transicionais na concentração mais elevada (1 µg/L). No gene *5-ht*$_{3B}$ verificou-se uma tendência de inibição crónica da sua expressão ao dia 21, contudo sem diferenças significativas. No sistema dopaminérgico, os genes do subtipo *d*$_2$ e *d*$_3$ após exposição do robalo à venlafaxina apresentaram aumento nos níveis de expressão no período de recuperação, na concentração mais elevada. Os antidepressivos partilham atividade farmacológica

comum, no entanto os nossos resultados demonstram que os SSRI e SNRI não partilham efeitos comuns nos genes que foram analisados, realçando um impacto adverso na expressão dos recetores do sistema de neurotransmissão. Sendo assim, os resultados apresentados fornecem novos desenvolvimentos sobre os efeitos crónicos dos antidepressivos quando em contacto com organismos marinhos não-alvos indicando diferentes mecanismo de toxicidade.

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List of abbreviations

µg	Microgram
5-HIAA	5-hydroxy-3-indolacetic acid
5-HIAL	5-hydroxy-3-indolacetaldehyde
5-HT_{3A}	Receptor of serotonin subtype 3A
5-HT_{3B}	Receptor of serotonin subtype 3B
5-HTP	5-Hydroxytryptophan
AADC	Aromatic L-amino acid decarboxylase
$ALDH_2$	Aldehyde dehydrogenase type 2
ANOVA	Analysis of variance
cAMP	Cyclic adenosine monophosphate
cDNA	Complementar Deoxyribonucleic Acid
CIIMAR	Centro Interdiciplinar de Investigação Marinha e Ambiental
CNS	Central Nervous system
DAG	Diacylglycerol
DAT	Dopamine Transporter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleotide
Dopamine D_2	Receptor of dopamine subtype D_2
Dopamine D_3	Receptor of dopamine subtype $D_{3\sim}$
DVEN	O-desmethylvenlafaxine
EDTA	Ethylenediamine tetraacetic acid
EF1	Elongation factor 1
FCT	Fundação para a Ciência e Tecnologia
FLUX	Fluoxetine
FSH	Folicle-stimulating gonadal axis
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GnRH	Gonodrotropin-releasing hormone
GPCR	G protein-coupled receptors
HPA	Hypothalamus-pituitary-adrenocortical
hpf	hours post fertilization
HPI	Hypothalamus-pituitary-internal
INFARMED	Portuguese National Authority for Medicine and Health Products
IP_3	Inositol triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside

K_{ow}	Octanol-Water Partition Coefficient
LC_{50}	Lethal concentration for 50% of the population
L-DOPA	L-3,4-dihydroxyphenylalanine
LH	Luteinizing hormone
LOEC	Lowest Observed Effect Concentration
MAO	Monoamine oxidase
MAOIs	Monoamine oxidase inhibitors
mRNA	Messenger Ribonucleic Acid
NCBI	National Centre for Biotechnology Information
NET	Norephinephrine Transporter
ng	Nanogram
NorFLUX	Norfluoxetine
OECD	Organization for Economic Co-operation and Development
PiP_2	Phosphatidylinositol 4,5-bisphosphate
PP	Psychopharmaceuticals
qPCR-RT	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SERT	Serotonin transporter
SNRI	Serotonin norepinephrine reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitors
TAE	Tris-acetate-ethylenediamine tetraacetic acid
TCA	Tricyclics antidepressants
TH	Tyrosine 3-monooxygenase or tyrosine hydroxylase
TPH	Tryptophan hydroxylase
VEN	Venlafaxine
VMAT	Vesicular monoamine transporter
WWTP	Wastewater Treatment Plants
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

Appendixes

Appendix I – Synthesis of samples types, country and concentrations reported of antidepressants

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Chapter 1

GENERAL INTRODUCTION AND OBJECTIVES

1.1. GENERAL INTRODUCTION

Emerging contaminants, such as active pharmaceuticals have been detected in wastewater, streams and drinking water (Thomas et al., 2012) due to inadequacy of wastewater treatment methods combined with low environmental degradability of some compounds, and excretion of unchanged parent compound. The term “emerging” is applied to compounds present in the waters on which very little is known about the potential impact on the environment (Deblonde et al., 2011). Such chemicals are a worldwide concern, not only for the human health but also due to potential effects on non-target species and in general to ecosystems. These environmental pharmaceuticals have specific effects on neurological and physiological systems in fish, amphibians, birds and mammals (Hampel et al., 2014; León-Olea et al., 2014), and their targets are highly conserved among vertebrates, and consequently aquatic animals, such as fish, are likely to be affected by environmental exposure (McGary et al., 2010).

In the last two decades, psychopharmaceuticals (PP), a huge group of pharmaceuticals, were recognized as an important source of environmental contamination. Selective Serotonin Reuptake Inhibitors (SSRIs) and Serotonin Norepinephrine Reuptake Inhibitors (SNRIs) are the most common classes within the PP and are used to treat depression, anxiety, obsessive-compulsive disorders, and panic disorders (Silverstone, 2004).

The main focus of this thesis was to evaluate transcriptional levels of genes in juvenile European Sea bass, *Dicentrarchus labrax (D.labrax)* (Linnaeus, 1758) brain in response to waterborne exposure to two PP, FLUX and VEN. Chapter 1 will provide insights into the main concepts addressed throughout this thesis in order to establish potential links between environmental exposures to PP and physiological and neurological changes in fish.

1.2. Worldwide consumption of antidepressants

Over the past few years, PP are recognized as an important source of environmental contamination and increasing concern has emerged regarding their potential ecological impact, associated to a higher prevalence of psychiatric disorders leading to an increased number of prescriptions for PP worldwide (Silva et al., 2012). Portugal has a high prevalence of mental diseases which the main responsible is depression (Furtado, 2012). Portuguese National Authority for Medicine and Health Products (Infarmed) reported central nervous systems (CNS) pharmaceuticals on the

2nd rank of medicine sales, among these the PP (SSRIs and SNRIs) (Furtado, 2012). Worldwide SSRIs and SNRIs are among the most prescribed PP (Schultz and Furlong, 2008), and reported increased consumption can be due to greater accessibility to drugs, prolonged use or the adoption of new therapeutic indications (Furtado, 2012). In 2020, depression is expected be the world's second most serious health disturbance (Lajeunesse et al., 2008). According to the last Eurobarometer published in 2010, 10% of EU citizens took antidepressants to treat mental health disorders during 2009 (Eurobarometer, 2010). The health data by the Organization for Economic Co-operation and Development (OECD) from 2011 reported that the use of PP in Portugal is higher (71.9%) than the EU average (52.5%), (Silva et al., 2012), emphasizing the importance of this study.

Comparing European countries between 2000 and 2012, Portugal had an increased consumption of antidepressants, higher than in Italy and Norway, but similar to Denmark. In 2000, the substance with the highest consumption was FLUX; however, during 2000 to 2012 a marked increase was observed for other SSRIs, such as sertraline and escitolopram. In the case of SNRIs, such as VEN, a similar high growth was reported, although the impact is lower compared to sertraline and escitolopram (Furtado, 2012).

1.3. Environmental presence of FLUX and VEN

Detection of PP began in the late 1990's and FLUX has been among the most ubiquitous pharmaceuticals found in environmental matrices (Schultz et al., 2011). FLUX is the PP with the most information available regarding behavior and physiological changes in aquatic animals, as well as data on the concentration in the environment as showed in Appendix I. Briefly, environmental concentrations of FLUX were found from 0.012 in United Stated (Kolpin et al., 2002) to 0.93 µg/L in Steinheim, German (Christensen et al., 2009) in sewage treatment plant discharges (WWTPs) and VEN from the low ng/L level to as high as 2 µg/L in wastewater effluent in Denver. VEN was the predominant antidepressant observed in wastewater and river water samples in a report by Schultz and Furlong, (2008).

Considering the presence of PP in aquatic ecosystems, the study of chronic effects of these PPs in aquatic species are needed to evaluate potential ecotoxicological risks, and to evaluate if aquatic organisms are affected by commonly observed, environmental concentrations.

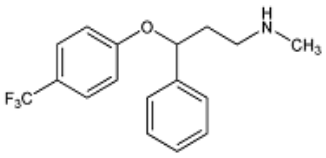
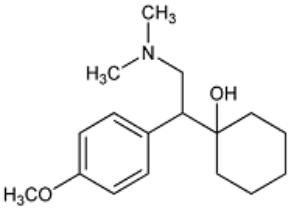
1.4. Antidepressants profiles

FLUX and VEN (Table 1) were both developed with the intention to alter the neurotransmitters biochemistry. Both compounds are polar, nonvolatile, non-biodegradable and are resistant to hydrolysis and photolysis (Bendz et al., 2005). Schlüsener et al. (2015) reports that increasing UV radiation leads to photo-degradation of the contaminants. Consequently, antidepressants tend to escape sedimentation and biological treatment in WWTP. Half-life of VEN in natural river water was 2.1 days and 19.2 h for their active metabolite Odesmethylvenlafaxine (DVEN) (Schlüsener et al., 2015); the half-life of FLUX is 1-3 days and their active metabolite norfluoxetine (NorFLUX) is 7-15 days (Airhart et al., 2007). FLUX and VEN are mainly biotransformed through *N-demethylation* by cytochrome *P-450* isoenzymes CYP2D6 (Mandrioli et al., 2006) in human liver, which results in the production of the active metabolite NorFLUX of FLUX (Hiemke and Härtter, 2000) and DVEN of VEN (Bisesi et al., 2014).

Their persistence increases the possibility of bioaccumulation in aquatic or terrestrial organisms, because they are moderately to highly lipophilic (Halling-Sørensen et al., 1998; Lajeunesse et al., 2011); they have by far the largest volume of distribution (up to 100 L/kg body weight), indicating extensive tissue accumulation (Kreke and Dietrich, 2008) and the compound octanol–water partition coefficient (Log K_{ow}) values > 3 are of great concern indicating that they can be adsorbed in soils and living organisms (Table I). The greater the Log K_{ow} of a compound, the more hydrophobic it is (Kreke and Dietrich, 2008). However, VEN was low or not observed (0.1 or 0.002 µg/kg) in brain tissue samples in wildlife fish (White sucker) sampled from two streams Creek in Colorado and Fourmile Creek in Iowa (Feito et al., 2013; Schultz et al., 2010). In fathead minnows when VEN and its metabolites were detected in tissues but did not accumulate to high concentrations (1.20 µg/kg) (Metcalfe et al., 2010). Thus, VEN levels in the neural tissue of fish suggested that this drug does not bioaccumulate during the exposure, although, as indicated above, the environment concentrations found were higher for VEN than FLUX antidepressant. In contrast with VEN, the FLUX antidepressant and it metabolite accumulated in the tissues (Kreke and Dietrich, 2008). Brooks and coworkers examined three different fish species, bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), and black crappie (*Pomoxis nigromaculatus*), from an effluent-dominated stream in northern Texas. In all three species, the highest concentrations of FLUX were detected in brain (1.58 ± 0.74 ng/g) and also in liver was detected FLUX (1.34 ± 0.65 ng/g), and the lowest concentrations were detected in muscle tissue (0.11 ± 0.03 ng/g) (Brooks et al., 2005). In Japanese medaka (*Oryzias latipes*) exposed to FLUX NorFlux concentration in brain was 5 times greater than FLUX (Nakamura et al., 2008). While the

FLUX concentrations in aquatic systems are well below concentrations that produce lethality, as determined by LC_{50} values (0.55 mg/L in mosquitofish, *Gambusia affinis*, to 1.6 mg/L in rainbow trout, *Oncorhynchus mykiss*), the bioconcentration of FLUX in the fish brain has raised concerns over potential sublethal effects on neuroendocrine modulation of physiological processes. These results document of antidepressants accumulated in aquatic organisms or uptake into brain tissue occurs from environmental exposure via water or food and/or bed sediment (Schultz et al., 2010). The lower observed concentrations of VEN in fish neural tissue may be explained in part by hydrophobicity, expressed by K_{ow}. The log K_{ow} of VEN is 3.28 which is lower than the reported than the log K_{ow} for FLUX, which are 4.05 (Table 1) (Schultz et al., 2010). Thusly, some poorly soluble pharmaceuticals have the potential to bioaccumulate, thus enrichment through the food chain is possible (Crane et al., 2006).

Table 1. Characteristics of FLUX and VEN

Chemical characteristics	**FLUX**	**VEN**
Structure		
CAS Number	54910-89-3	99300-78-4
Molecular weight (g/mol)	309.33 (Budavari, 2006)	277.4 (Budavari, 2006)
Empirical formula	$C_{17}H_{18}F_3NO$ (Budavari, 2006)	$C_{17}H_{27}NO_2$ (Budavari, 2006)
***log K*$_{ow}$**	4.05 (Nentwig, 2007)	3.28 (Rúa-Gómez and Püttmann, 2012)
Solubility in water (mg/g)	38 (Nentwig, 2007)	270 (Rúa-Gómez and Püttmann, 2012)

1.5. Pharmacology of SSRIs and SNRIs

The mechanisms of action of SSRIs and SNRIs are similar. SSRIs act in the serotonergic system of the central nervous systems (CNS) by inhibiting the reuptake of a serotonin by their carriers (serotonin transporter or *sert*) in the presynaptic membrane. Thereby increasing the concentration of serotonin in the synaptic clefts, increasing the serotonergic neurotransmission (Kreke and Dietrich, 2008; Valenti et al., 2012). SNRIs act not only in serotonergic system, but in three neurotransmitters: serotonin, like SSRIs, norepinephrine and a somewhat weaker inhibition of dopamine (Fenli et al., 2013; Yamamoto and Vernier, 2011) and are approximately 10 times more potent at inhibiting serotonin uptake than norepinephrine (Celikyurt, 2012).

The monoamine serotonin and the catecholamines, dopamine and norepinephrine, are concentrated and stored within localized vesicles in axons, dendrites and cell bodies (Golan et al., 2012). In vertebrates these neurotransmitters play essential roles in several physiological processes (Barros et al., 2012; Teyke et al., 1993). The storage process of neurotransmitters is performed by the vesicular monoamine transporter (*vmat*) – a non-specific monoamine transporter – that is released at the synapse following neuronal membrane depolarization. The serotonin, dopamine and norepinephrine from the synaptic cleft is than recycled by *sert*, dopamine transporter (DAT), norepinephrine transporter (NET) respectively, and this process represents the first important step in the of transmitter recycling. When the monoamines are not stored in presynaptic vesicles by *vmat*, they are degraded by monoamine oxidase (*mao*) enzymes located in the outer mitochondrial membrane (Maximino, 2012). The *mao* is present in two isoforms in mammals, *mao a* and *mao b*, with the first having higher affinity for serotonin (Johnson, 1968). After the release of monoamines to the synaptic cleft, the activation of receptors occurs postsynaptically, and the neurons opens ligand gated ion channels (*5-ht*$_3$), which regulate the action potential, and therefore, the signal transmission in neurons (Bisesi, 2014).

1.5.1. Monoamine serotonin

The monoamine serotonin or 5-Hydroxytryptamine is a neurotransmitter with a wide range of functions that is found in both the central and peripheral nervous systems (Jain, 2002). It was initially isolated and identified by Rapport and colleagues in 1948 (Mennigen et al., 2011; Rapport et al., 1948). It is now known to exist in nearly every biological organism, including plants, invertebrates and vertebrates, which indicate an evolutionarily ancient origin. For curiosity, this neurons system of the mammalian brain comprise the most extensive and complex neurochemical network in the CNS after that

of glutamate, which makes up the basic wiring of the brain. It has been estimated that the human brain contains about 250 000 serotonin neurons of a total of 10^{11} neurons (Artigas, 2012). In vertebrates, high concentrations of serotonin are found in the brain, gut, kidney and lung (Kreke and Dietrich, 2008; Mennigen et al., 2011). Furthermore, serotonin is present in the serum, and has effects on smooth muscle contraction (Kahn et al., 1992). The majority of monoamine serotonin is found in the gut, where it modulates motility and initiates peristaltic and secretory reflexes (Gershon, 2003). Serotonergic projections exist from the raphe nuclei to almost every part of the vertebrates brain; areas as thalamus and cortex (Howe et al., 2013; Larson et al., 2014). In mammal's brain, serotonin is a key neurotransmitter that modulates normal physiology including sleep, food intake, stress (Larson and Summers, 2001), appetite, the immune system, reproduction, behavior (Brooks et al., 2003) and sexual behavior (Lucki, 1998). Serotonin has the same function in vertebrates, including the regulation of locomotion, mood control (Veenstra-VanderWeele et al., 2000), feeding (Larson and Summers, 2001), brain development (Cornide-Petronio et al., 2015; Daubert and Condron, 2010), energy metabolism (which depends on complex interaction between many physiological and environmental factors) (Pérez Maceira et al., 2014) and aggression (Huber et al., 1997; Larson and Summers, 2001; Raleigh et al., 1991), such as FLUX, have been found to inhibit food intake (Pérez Maceira et al., 2014).

In chemical terms, this neurotransmitter is a hydrophilic indole amine derived from the amino acid tryptophan. The key enzyme for serotonin synthesis is the tryptophan hydroxylase (TPH), present in the brain mostly in its second isoform, TPH_2, which converts tryptophan to 5-hydroxytryptophan (5-HTP), a process limited by tryptophan availability (Maximino, 2012). 5-HTP is than conversed in serotonin by the aromatic L-amino acid decarboxylase (AADC). Both enzymes are located in the cytoplasm of serotonergic neurons in both the cell body and the cellular processes. The principal enzyme in monoamine degradation is MAO, the oxidative deamination of serotonin by converting it into 5-hydroxy-3-indolacetaldehyde (5-HIAL), which is further metabolized into 5-hydroxy-3-indolacetic acid (5-HIAA) by aldehyde dehydrogenase type 2 (ALDH2) (Pytliak et al., 2011).

1.6. Catecholamine: Dopamine and Norepinephrine

Dopamine and norepinephrine belongs to the family of neurotransmitter catecholamine and are widely distributed in mammalian species and vertebrates. Dopamine constitutes about 80% of the catecholamine content in the brain (Vallone et al., 2000). Dopamine and norepinephrine act as modulator of neuronal activity

regulating many different functions in CNS (Callier et al., 2003; Park et al., 2006). Vertebrates (Cooper et al., 2003) and mollusks (Lacoste et al., 2001) release norepinephrine during acute stress in response to danger (Adamo, 2008; Wingfield, 2003). The release of norepinephrine during acute stress and their responses prepare the animal to physiological changes that can influence their immune function (Adamo, 2008). Dopamine exerts its physiological systems or organs by binding to multiple membrane receptors couple to heterotrimeric G proteins (GPCR) (Callier et al., 2003). It is described to be involved in the modulation of a number of key cerebral functions, as sensory perception, learning and memory, hormonal regulation and control of body temperature among others (Blackstone, 2009; Yamamoto and Vernier, 2011). Similar to serotonin, dopamine controls food intake and sexual behavior (Callier et al., 2003). Central dopaminergic neurons originate, for the most part, in different areas of the brain and have different projections.

Catecholamines are amine derivatives of catechol (2-hydroxyphenol) (Yamamoto and Vernier, 2011).synthesized from L-3,4-dihydroxyphenylalanine (L-dopa) by the action of aromatic amino acid decarboxylase (AADC), which is produced from tyrosine by tyrosine 3-monooxygenase or tyrosine hydroxylase (TH). Following, dopamine is further processed in norepinephrine by dopamine ß-hydroxylase and subsequently in epinephrine (or adrenaline) by phenylethanolamine N-methyltransferase (Golan et al., 2012).

1.6.1. Biogenic amine receptors

Serotonin receptors are divided into seven different families (*5-ht$_1$, 5-ht$_2$, 5-ht$_3$, 5-ht$_4$, 5-ht$_5$, 5-ht$_6$ and 5-ht$_7$*) (Hoyer et al., 2002) based on the receptor structure, their affinity for different ligands and activation of the secondary messenger (Chegini et al., 2014). In teleost fish, serotonin receptors have been identified and characterized in several species such as zebrafish (*Danio rerio*), European flounder (*Platichthys flesus*), Gulf toadfish (*Opsanus beta*), and Puffer fish (Yamaguchi and Brenner, 1997; Lu et al., 2007; Best and Alderton, 2008; Mager et al., 2012). Serotonin receptors comprise multiple subunits, and the G-protein coupled (metabotropic receptors) signaling pathways are of the Gs, Gq and Gi sub-types, with the exception of the *5-ht$_3$* receptor that are ligand-gated ion channels (ionotropic) (Pytliak et al., 2011). The *5-ht$_3$* receptor directly gates an ion channel inducing rapid depolarization and consequently the release of neurotransmitters (Artigas, 2012; Kondaurova et al., 2012). This ion channel receptor are members of the Cys-loop superfamily with relatively conserved trans

membrane domains (Descarries and Riad, 2012), and comprises two subunits known as *5-ht$_{3a}$* and *5-ht$_{3b}$* cloned in Human (Chameau and Van Hooft, 2006). Until now, five dopamine receptors proteins were isolated in vertebrates encoded by different genes. Along the years, there was a strong interest for dopamine receptors due to the alteration in dopamine transmission in a few human pathologies, essentially Parkinson's disease, addiction to drugs of abuse, as cocaine, or disorders of mood and schizophrenia (Callier et al., 2003). In vertebrates different receptors mediate dopamine signaling, D_1–D_5. These receptors are grouped into two classes (D_1-like and D_2-like) dependent on the G-protein to which they couple, the basis of their differences in biochemical and pharmacological properties. The presynaptic dopamine receptor, most of which belongs to the class D_2 (D_2, D_3 and D_4), act as auto receptors. These auto receptors perceive excessive flow of dopamine from the synapse and reduce the dopaminergic tone, reducing the dopamine synthesis in the presynaptic neuron and reducing neuronal discharge rate and the release of dopamine (Callier et al., 2003).

1.7. European seabass (*Dicentrarchus labrax*): marine fish in study

European seabass belongs to the order of *Perciformes*, family *Moronidae* (Vázquez and Muñoz-Cueto, 2015), and is a gonochoristic marine teleost fish (Louro et al., 2014). European seabass is an important popular cultured specie with a high economic value (Santos et al., 2010). In Europe, the consumption of Sea bass and production demand has increased over the past 15 years (Fuentes et al., 2010), and nowadays is the fourth most produced fish in European aquaculture (Tine et al., 2014). For this reason, its production was expanded along the Mediterranean coast, and Sea bass became one of the main cultured fish species (Fuentes et al., 2010). Due to high capture pressure, future conservation and management issues were raised (Tine et al., 2014). The specie is distributed along the northeast coast of the Atlantic Ocean, from Norway to Senegal and from Mediterranean to Black Sea (Kottelat and Freyhof, 2007; Vázquez and Muñoz-Cueto, 2015), being also found throughout the Portuguese coast (Vasconcelos et al., 2009).

Due to accumulation of contaminants in several tissue, including in muscle, the marine fish can be a vehicle for human exposure to compounds (Fent et al., 2006; Kreke and Dietrich, 2008; Lajeunesse et al., 2011; Reis-Henriques et al., 2009). Also, *D. labrax* are carnivorous, feeding on fish, crustaceans and cephalopods(Pickett and Pawson, 1994)(Pickett and Pawson, 1994) and, may accumulate organic pollutants through its food (Dural et al., 2007; Türkmen et al., 2005). Moreover, the European Sea

bass is described has a good bioindicator organism reflecting the local water environmental pollution in their tissues (Schnitzler et al., 2011).

This marine species is a euryhaline fish tolerating a range of salinities (0-60 psu) (Tine et al., 2014). Most studies with aquatic organisms exposure to contaminants is based on freshwater species (Bisesi et al., 2014; Brooks, 2014; Brooks et al., 2003; Kreke and Dietrich, 2008; Metcalfe et al., 2010). So, it is essential to analyze the effects in a marine fish species, which have striking differences in their physiology (e.g. osmoregulation). In 2014 with seabass genome published it was proven that the European Sea bass genome has the highest number of gene copies linked to ion and water regulation, with 94 genes, among fully sequenced teleost (Tine et al., 2014).

A decade ago, few fish genomes were available *Fugu rubripes* (Aparicio et al., 2002), *Tetraodon nigroviridis* (Jaillon et al., 2004), *Danio rerio* (Howe et al., 2013), *Oryzias latipes* (Kasahara et al., 2007) and *Gasterosteus aculeatus* (Jones et al., 2012). The European Sea bass moved in the last 10 years to the forefront of availability of genetic and genomic resources (Louro et al., 2014), and since 2014 the genome is publically available (http://Sea bass.mpipz.mpg.de, Tine et al., 2015). Hence, the species is a good experimental model for ecotoxicological assays, due to their important role in their ecosystems, wide geophraphical distribution, availability through aquaculture production and the good response in behavioral terms when exposed to pollutants (Almeida et al., 2010). European Sea bass also constitutes an important model for many basic research areas, including population genetics (Bahri-Sfar et al., 2000), feeding activity (Sánchez-Vázquez et al., 1998), response to salinity (Boutet et al., 2006; Nebel et al., 2005). However, there is a need for more information on contaminant levels in European fish and on chronic effects due to exposure to emerging pollutants.

1.8. Overview of the effects of antidepressants

The scarcity of information about the fate and the long-term effects of PP and their active metabolites in aquatic species makes risk assessment difficult (Lajeunesse et al., 2011). Futhermore, the impacts and ecotoxicological potencial of neuroactive drugs on aquatic organisms and communities are important to assess (Bendz et al., 2005), but are still not well understood or described (Kreke and Dietrich, 2008; Lajeunesse et al., 2011).

There are four important reviews (Brooks et al., 2003; Kreke and Dietrich, 2008; Menningen, 2011; Oakes et al., 2010) and a more recent serotonin review (Prasad et al., 2015) that provide several antidepressants-induced neuroendocrine disruption in

teleost fish, for example in reproduction and endocrine functions, control of feeding, fish stress, behaviors and immune system. In the reproduction and endocrine function in teleost fish, the monoamine serotonin can affect gonadotropin-releasing hormone (GnRH) regulated gonadotropin release from the pituitary (Khan and Thomas, 1994). So, the regulation of reproduction by hypothalamo-pituitary-gonadal (HPG) axis and it is stimulated by GnRH, which act to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary (Prasad et al., 2015), both of which can regulate key genes involved in reproductive physiology including sex hormone synthesis, oogenesis, and vitellogenesis (Khan and Thomas, 1994).

Through modulation of neurotransmitter systems by exogenous compounds, reproductive fitness can be altered via effects on behavior and physiology. PP altered the predator avoidance behavioral phenotype of juvenile fathead minnows (Thomas et al., 2012) due to alterations in brain function that impair predator avoidance behavior caused by changes in neurotransmitter levels in the brain. In most vertebrate species examined so far, increased serotonergic activity has been associated with an inhibition of aggressive behavior (Kreke and Dietrich, 2008). Behavioural effects of FLUX and VEN has been observed in larval fathead minnows and hybrid striped bass including a reduction of the escape response, a measure of predator avoidance (Yamaguchi and Brenner, 1997).

It has long been recognized that stress affects the immune response in mammals (Kreke and Dietrich, 2008). An investigation reported that serotonin may play a role as modulator of the immune response in fish and that SSRI may mimic immunomodulatory effects. It has been shown that isolated lymphocytes cells from rainbow trout (Oncorhynchus mykiss) may express a functional SERT that can be blocked by SSRIs, thereby inhibiting serotonin uptake into the cells (Ferriere et al., 1999).

1.9. Objectives

The general aim of this master thesis was to evaluate transcriptional levels of genes in a marine fish species, the *D. labrax*, in central nervous system in response to waterborne exposure to two PP. Firstly, selected target genes of the European Sea bass were identified and characterized from the neurotransmitter system in the brain. Secondly, European Sea bass was chronically exposed to two selected antidepressants and chronic effects were analyzed by qPCR-RT on the transcriptional patterns of neurotransmitter genes from the brain.

In detail the objectives of this study were:

(i) To identify selected target genes of the European seabass from the neurotransmitter system in the brain. To compare selected target sequences of European Sea bass to sequences of other species *Oryzias latipes*, *Takifugu rubripes*, *Homo sapiens* and *Danio rerio*, by multiple alignments, and phylogenetic trees.

(ii) To design and evaluate real-time PCR assays for genes of interest, such as 5-Hydroxytryptamine subtype *5-ht*$_{3a}$ and *5-ht*$_{3b}$, serotonin transporter receptor (*sert*), monoamine oxidase (*mao*), vesicular monoamine transporter (*vmat)* and dopamine d_2 and dopamine d_3;

(iii) To analyze effects on the gene expression in European Sea bass chronically exposed to two selected antidepressants, FLUX (SSRI) and VEN (SNRI).

The selected targets for the gene expression analysis were based on the following rationale: (i) The serotonin and dopamine receptor subtypes are important, because the monoaminergic system of the CNS is generally well conserved in vertebrates, and monoamines and catecholamines are implicated in the regulation of feeding, growth, reproductive fitness and others; (ii) The monoamine oxidase controls the degradation of monoamines in presynaptic cleft; (iii) The neurotransmitter transporters (*sert* or *dat*) represent the main targets for the pharmacological action of SSRIs or SNRIs. These transporters are located in the presynaptic plasma membrane and are primarily responsible for the synaptic clearance of serotonin or dopamine after neurotransmitter release.

Overall, the presented findings contribute to the study of chronic exposure effects of PP on a marine fish species, which may be used as a sentinel species in estuaries or coastal regions.

1.10. Thesis structure

This thesis is organized in three chapters: the first includes a general introduction to the topic under investigation, and to the objectives of the study. The second chapter follows in general the structure of a research paper including a brief introduction, material and methods, results, discussion, and conclusions. The third and final chapter includes a general discussion, the final conclusions and references.

Chapter 2

TRANSCRIPTIONAL LEVEL OF GENES INVOLVED IN THE NEUROTRANSMITTER SYSTEM OF *DICENTRARCHUS LABRAX* IN RESPONSE TO CHRONIC EXPOSURE TO PSYCHOPHARMACEUTICALS

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Transcriptional level of genes involved in the neurotransmitter system of *Dicentrarchus labrax* in response to chronic exposure to psychopharmaceuticals

Abstract

Up to 10% of the human population suffers from depression. SSRIs and SNRIs are prescribed for clinical depression and detected in aquatic ecosystems. The main aim of this study was to explore and evaluate transcriptional levels of neurotransmitter genes in brain of a marine fish species European sea bass. The juveniles were exposed to two PP: (i) FLUX at the concentration of 0.5 µg/L and 50 µg/L; (ii) VEN at the concentration of 0.01 µg/L and 1 µg/L. The chronic exposures were performed for 21 days, and followed by a 7 day of recovery period to assess the reversibility of effects. Transcription levels of genes encoding for proteins involved in the neurotransmitter system, such as *5-ht$_{3a}$ and 5-ht$_{3b}$, sert, mao, vmat* and dopamine *d$_2$* and *d$_3$* were analyzed using qPCR-RT.

One of the main results was that both antidepressants act differentially on the neurotransmitters gene expression. We found strong and unique profile of effects in transcription levels in FLUX and VEN. The FLUX exposure revealed acute induction on day 1 in all targets genes, except *mao*, where a chronic effect was present at day 21. In contrast to FLUX, VEN exposure at day 21 decreased the mRNA expression of the receptors of serotonin *5-ht$_{3a}$* and *5-ht$_{3b}$*, but only for *5-ht$_{3a}$* with significant differences. In addition, VEN exposure increased Dopamine *d$_2$* and *d$_3$* receptor mRNA expression at the recovery period at day 28.

Analyzed dopaminergic and serotonergic neurotransmission genes from European Sea bass had a high degree of homology with *D.rerio, O.latipes, T.rubripes*, and *H. sapiens*, analyzed by multiple alignments and phylogenetic trees. Sequence similarities indicate the possibility of similar gene functions in vertebrates. Modulated genes from this study, are associated in humans with several mental disorders, and are known to control various behavior and food intake in aquatic vertebrates. These results provide new insights into the chronic effects of psychopharmaceutical compounds and indicate different mechanism of toxicity.

Keywords: *Fluoxetine; Venlafaxine; European seabass; qPCR-RT; serotonin receptors; dopamine receptors; neurotransmission; neurotransmitters*

2.1. INTRODUCTION

Since the first detection of pharmaceuticals in sewage water effluents in the mid-1970s, there have been numerous reports on the presence of these substances and their metabolites in surface waters on a global scale (Grabicova et al., 2014a). The wide distribution of these substances in the environment has become a high priority for regulatory agencies involved in human and ecological risk assessment (Park et al., 2012). The biological activity of some pharmaceuticals indicates that they can have physiological effects on non-target organisms at environmental relevant concentrations (Brooks et al., 2003; Lajeunesse et al., 2011; Mennigen et al., 2011). Within the pharmaceuticals a major group exists – in terms of medicine sales and increasing consumption – where scarce information is available, the PP (Santos et al., 2010). This group includes monoamine reuptake inhibitors, e.g. selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine (FLUX), and serotonin and norepinephrine reuptake inhibitors (SNRIs), such as venlafaxine (VEN) (Grabicova et al., 2014b). It has long been understood that neurotransmitters, especially monoamines, play a major role in modulation of behavior, as well as many physiological functions in humans. Serotonin and dopamine are two of the major players of the neurotransmitter system (Gardner et al., 2008). Serotonin has a critical role in the regulation of temperature, behavior, appetite, sleep and mood control. Dopamine has been implicated in the control of motoric function and emotional behavior (Schultz, 2002). Whereas norepinephrine is thought to be involved in behavioral arousal, as well as playing a major role in fight or flight response (Harley, 2004). The mechanisms of action of SSRIs (e.g FLUX) and SNRIs (e.g VEN) are similar: SSRIs act on the serotonergic system of the CNS by inhibiting the reuptake of serotonin by their carriers (SERT) in the presynaptic membrane, thereby increasing the concentration of serotonin in the synaptic clefts and increasing serotonergic neurotransmission (Kreke and Dietrich, 2008; Valenti et al., 2012); SNRIs act not only on the serotonergic system, but on three neurotransmitters: serotonin, norepinephrine and a weaker inhibition of dopamine (Fenli et al., 2013; Yamamoto and Vernier, 2011). Because monoamines directly modulates some cellular functions and acts on the CNS in many organisms (Kreke and Dietrich, 2008), manipulations of monoamines levels can influence diverse physiological processes, as reproduction, development, behavior and neuroendocrine signaling pathways (Brooks et al., 2003; Foran et al., 2004; Kreke and Dietrich, 2008; Mennigen et al., 2011; Oakes et al., 2010). Previous studies indicate that aquatic species exhibit abnormal behavior and lower levels of anxiety when they are chronically exposed to SSRIs impeding fitness and survival on a population scale

(Almeida et al., 2012; Bisesi et al., 2014; Brooks et al., 2003; Kreke and Dietrich, 2008; Weinberger and Klaper, 2014). Generally a high level of serotonin activity is associated with low levels of aggression, apprehensive and/or subordinate behavior. In contrast, individuals with low serotonin function tend to be more aggressive, bold and/or have a dominant position and an increased time to capture prey (Oakes et al., 2010). Although, the question of how gene expression is perturbed in the brain by antidepressants exposure is not yet explained. The gene expression analysis in aquatic species can elucidate the alterations of transcription levels, detect effects of contaminants on biochemical pathways, and be used for comparison of gene expression profiles to determine differences/similarities in responses of several organisms (Park et al., 2012; Rogers et al., 2011). There are no studies comparing effects of different classes of pharmaceuticals, as SSRIs and SNRIs on global gene expression in aquatic organism.

The main focus of this study was to evaluate transcriptional levels of genes in juvenile European seabass brain in response to waterborne exposure to two PP, FLUX and VEN. Two different concentrations were chosen, one orientated to frequently observe environmental level, and the other to rather physiological relevant level (100x higher than environmental level). To answer the question whether PPs act differentially on the neurotransmitters, transcription levels were measured in brain by quantitative real-time qPCR-RT. Since the major focus of previous works was on freshwater species, in this work we have chosen a marine fish species, one first study with a marine organism, which have striking differences in their physiology (e.g. osmoregulation) (Tine et al., 2014). A wide geographic distribution over Europe and is explored as a sentinel species for environmental field studies covering estuaries and marine coastlines. Moreover, Sea bass is an important cultured species with economic value, and changes in aggression or feeding behavior can have a negative impact in aquaculture production in estuaries. We discuss these findings in the context of transcription of levels in neurotransmitters in qPCR-RT because FLUX and VEN has been detected in the environment. The environmental relevance of PP exposure and their chronic effects on *D.labrax* were analyzed and will be helpful in designing methodologies for future risk assessments of pharmaceuticals that target the neuroendocrine system.

2.2. MATERIAL AND METHODS

2.2.1. Fish maintenance

Juvenile European Sea bass were obtained from a commercial aquaculture (Aquanord, Gravelines, Nord, France), and kept at the Aquatic Animal Facility of CIIMAR – Interdisciplinary Centre of Marine and Environmental Research (Portugal, Porto). Fish were acclimated for at least 1 month before the beginning of the experiments. Animals were kept in tank with 2000 L; they were fed every day and kept under natural conditions of temperature (13.5 ºC-16.5 ºC) and range of salinities (22.4 - 25.7 psu). In our facilities, we kept Sea bass at pH between 5.78-6 in tanks with artificial reconstituted seawater. Levels of ammonia and nitrite were checked daily and maintained below 0.5 mg/ml, with value range 0.02 - 0.04 and 0.06 - 0.07, respectively.

2.2.2. Fish condition and exposure

The European Sea bass was exposed to two antidepressants FLUX and VEN in a flow-through system in two different experiments. Two concentrations were chosen for each PP: 0.5 µg/L and 50 µg/L for FLUX, 0.01 µg/L and 1 µg/L for VEN. After the acclimation period, the European seabass was exposed in 12 tanks with 61 L each, with a water daily replacement of 70%. For each treatment, 3 replicates of the following groups control; solvent control group (dimethyl sulfoxide – DMSO 0.008%); FLUX with 0.5 µg/L and 50 µg/L. In the experiment with VEN, the same experimental setup was used, three replicates per group control; solvent control group (DMSO) with concentration 0.01%; VEN at 0.01 µg/L, and 1 µg/L. A total of 288 animals were used for the exposure experiment with FLUX, and 144 seabass for the exposure experiment with VEN; animals were randomly distributed in the aquariums (24 animals in each aquarium in the FLUX experiment, and 12 animals per aquarium in the VEN experiment). The exposure lasted for 21 days (chronic exposure) and followed by a 7 day period of recovery (no compound or DMSO added) (Appendix II). The animals were fed during the experiments every 2 days.

2.2.3. Tissue sampling

Biological sampling was performed at day 1 and day 21 of exposure, and at day 28 after the recovery period (Fig. 1). For tissue sampling, animals were anesthetized in MS222 (0.25%). Before dissection, seabass was weighed and measured (total and standard) and the cranial region was dissected. The total brain were excised from the

head, placed in RNA Later at 4 ºC overnight and stored at -80 ºC until further use for gene expression analysis.

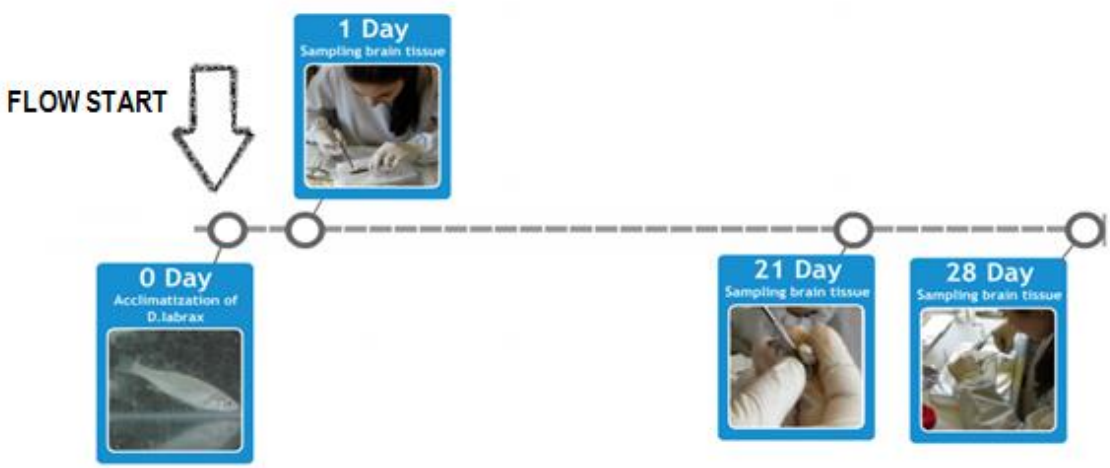


Fig. 1. Scheme chronological dissection of tissues. Animals were added to the aquarium for acclimatization. At day 0 the experiment started by addition of the compounds. At day 1 (one day after adding the compounds) we did the first sampling. The last 7 days no release contaminants or DMSO.

2.2.4. Design of primers

2.2.4.1. Database construction and annotation

Primer pairs for real-time PCR for 5-Hydroxytryptamine subtype 3A and 3B (5-HT_{3A} and 5-HT_{3B}), serotonin transporter receptor (*sert*), monoamine oxidase (MAO), vesicular monoamine transporter (*vmat*) and dopamine d_2 and d_3 (Table 2) were designed based on the genome sequences of European seabass, which was recently published by Tine, 2014 (Tine et al., 2014), available at the UCSC Genome Browser at http://Sea bass.mpipz.mpg.de/cgi-bin/hgGateway.

After obtain the Sea bass mRNA sequences, we compared them to the DNA sequences. Using the comparison to DNA sequences, specific primers were designed that were spanning an intron, such that primers were located on different exons (Appendix IV). For primer design we used the software PRIMER 3 Plus Primer (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/).

Important parameters for the design of primers are the annealing temperature, % GC (50%) and the localization of the primer – influencing their efficiency. The oligonucleotides were purchased from Stabvida (Portugal).

2.2.5. RNA extraction and agarose gel electrophoresis and quantification

Total RNA was isolated from approximately 30 mg of tissue samples using the RNAspin Mini Kit (GE Healthcare, Hilden, Germany) following the manufacturer's instructions. RNA quality was checked by gel electrophoresis on a 1.5% agarose gel (NZYtech genes and enzymes, Lisbon, Portugal). In the extracted RNA of brain samples from Sea bass, two major bands were verified corresponding to the 18S and 28S subunits (Fig.2). RNA concentration was determined using a UV-Vis spectrophotometer measuring the 260/280 ratio, *Take3™ Multi-Volume Plate* (Biotek, Taiwan) and the plates with volume 2 µl (Biotek, Taiwan). Total RNA was extracted and verifying the integrity and quality of RNA in agarose gel bands. Following, 1 µg of total RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen). In Appendix VI shows the difference of RNA quality.

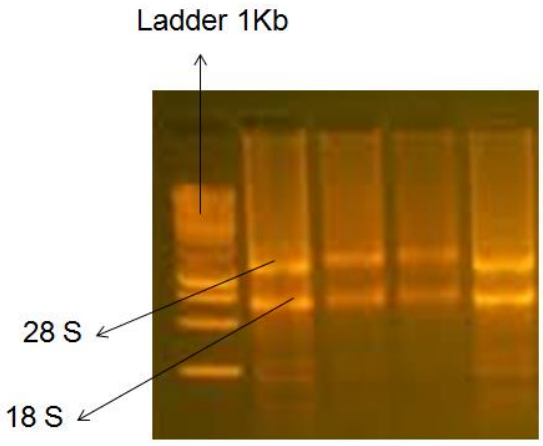


Fig. 2. Electrophoresis on agarose gel (1.5%) corresponding bands of the 28S and 18S juvebile Sea bass brain in brain RNA samples

2.2.6. cDNA synthesis and Polymerase chain reaction (PCR)

cDNA was synthesized using the iScript cDNA Synthesis Kit from BioRad. To confirm the sequences identities, PCR was performed in a Biometra Thermocycler with 1 µl of cDNA, 0.2 mM of DNTPs, 0.4 µM of each primer and 2.5 mM of MgCl2 in a total volume of 25 µl, using *Taq DNA Polymerase* (BioRad, EUA). The protocol was performed using the following conditions: 2 minutes denaturation at 94 ºC, 30 second denaturation at 94 ºC, 30 seconds annealing at 55 ºC, and 20 seconds at 72 ºC of

extending the polymerization reaction, for 35 cycles, followed by 2 minutes of a final extension at 72 ºC.

2.2.7. Electrophoresis agarose gel, DNA isolation, cloning and sequence analysis

PCR products were separated by gel electrophoresis on a 1.5% agarose gel in Tris-acetate-EDTA buffer – TAE buffer stained with 6x GelRed™ Nucleic Acid (Biotium) and the PCR products were visualized under UV light. The bands of expected size were cut out carefully and eluted from the gel according to manufacturer´s protocol, using the commercial Gel Band Purification Kit, GE Healthcare. It was observed with high resolution gel electrophoresis and resulted in a single product with length 109bp sequence similar to SERT, a 91bp sequence similar to 5-HT_{3B}, a 186bp sequence similar to 5-HT_{3A}, a 88bp sequence similar to D_3, a 121bp sequence similar to D_2, a 86bp sequence similar to VMAT and a 100bp sequence similar to MAO were identified (Appendix III).

The isolated fragments were then inserted in *pGEM* plasmid vector (pGEM® - T Easy Vector Systems – Promega) and incorporated in *E. coli* using Blue Competent Cells (Novagen). Bacteria were grown overnight for 16-24 hours in solid medium (40g/L LB Agar (nzytech), ampycilin 100 µg/ml, Isopropyl β-D-1-thiogalactopyranoside (IPTG) 0.5 mM and X-Gal 40µg/ml) at 37 ºC. The correct colonies were isolated (white colonies) from solid medium and incorporated in 5 ml Liquid medium LB Broth (20g/L, Sigma) and cultured overnight at 37 ºC with shaking. After, the plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega). The inserts were sent to STABVIDA (Portugal) for sequencing. The identities of all sequences were checked using the Basic Local Alignment Search Tool (Blast) at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), after using the Vecsreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen) to identifying segments of a nucleic acid sequence that may be of vector origin. The alignments of the obtained sequences with other mammal and fish species was conducted with ClustalW Multiple Alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Phylogenetic trees were performed with the free software Mega 6.06 (http://www.megasoftware.net/). Phylogenetic analysis was done using the neighbor-joining method and a percentage of concordance based on 1000 bootstrap iterations.

2.2.8. Real-Time quantitative qPCR-RT

Gene expression quantification of SERT (002060), $5HT_{3A}$ (00148200), $5HT_{3B}$ (00046560), D_2 (00129960), D_3 (00076420), MAO (00054530), VMAT (00018080) was performed in brain from Sea bass by means of quantitative real-time reverse transcriptase PCR (qPCR-RT). Elongation factor 1 (*EF1*), 18S ribosomal RNA (*18S rRNA*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein L17 gene (*L17*) were evaluated as possible reference genes.

For qPCR-RT, 1 µg of total RNA was subjected to digestion of genomic DNA using DNAse I and first strand cDNA was synthesized using iScript cDNA Synthesis Kit from Biorad. Optimal primers concentrations were determined after evaluation of the highest fluorescence signal at lower Ct number. The concentrations tested were 600 nM, 300 nM, 150nM and 50nM; finally 300 nM was selected for the amplification of target and housekeeping genes. Real-time PCR amplification was done in an Eppendorf Realplex 4, with the plates calibrated with iCycle IQ® PCR plates. Also, use IQSybr Green Supermix (Bio Rad), with 10 µl SYBR Green mix, 2 µl of each primer (final concentration of 300 nM) and 1 µl of cDNA in a total volume of 20 µl. Reactions were conducted under the following conditions: 95 ºC for 3 minutes, followed by 40 cycles at 95 ºC for 10 seconds, 54 ºC for 30 seconds and 72 ºC for 30 seconds. At the end of each run, a melting curve analysis was done (from 55 ºC to 95 ºC) to determine the formation of specific products. Samples were run in technical duplicates. Controls (no template added) were run to exclude contamination and the formation of primer dimers. To determine the efficiency of the PCR reactions, standard curves were made for all the genes, with 6 serial dilutions of the template (concentrations range from 0.05 to 50 ng/µl), and the slopes and regression curves were calculated. Quantification of the mRNA expression of the genes were normalized with a multiple reference gene approach as detailed in 2.1.10.

Melting curve analysis was performed which resulted in single product specific melting temperatures. No primer-dimers were generated during the applied 40 real-time RT-PCR amplification cycles and in control of No Template Control (NTC) were not identified expression samples.

2.2.9. Confirmation of primer specificity and Real-time PCR amplification efficiencies

Real-time RT-PCR efficiencies were calculated from the given in real-time RT-PCR software. Although, to confirm the software efficiency it was calculated according

to the equation: $E=10^{(-1/slope)}$ (Table 2; Fig.3) and threshold was defined for all genes at 100 units of fluorescence intensity.

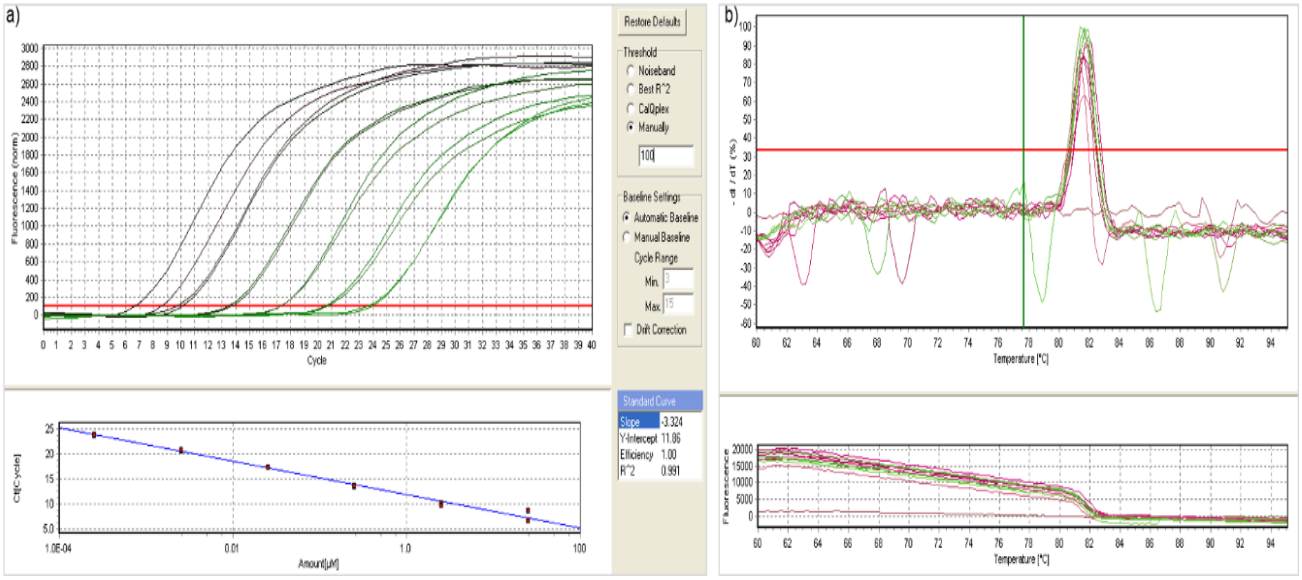


Fig. 3. Real-time RT-PCR SYBR Green fluorescence of *5-ht$_{3b}$* gene with: a) efficiency curve, slope value and threshold value (100); b) melting curve respectively.

2.2.10. Normalization of reference genes

The most commonly used normalization strategy for quantitative real-time qPCR-RT, involves standardization to a constitutively expressed control gene. The normalization is necessary in all experimental conditions. No single gene is constitutively expressed in all cell types implying that the expression stability of the intended control genes has to be verified before each experiment (Andersen et al., 2004). The expression of the target genes (*sert, 5-ht$_{3a}$ and 5-ht$_{3b}$, dopamine d$_2$ and d$_3$, mao and vmat*) was normalized to the mRNA expression of multiple reference gene apllying the Normfinder (Andersen et al., 2004). In the case of Normfinder, the software calculates the average expression stability between four selected potential reference genes (*GAPDH, L17, EF1, 18s*). The best combination of two reference genes was selected (Fig.4), which was the combination of *18S* + *EF1* (stability value: 0.654) in our exposure experiments to FLUX and VEN. The intergroup variations plotted with the intragroup variation as error bars (Fig. 4) is given an overview of the obtained expression stability for all analyzed reference genes as calculated by NormFinder. For the software program (Normfinder), all samples from the corresponding treatment groups and sampling dates used to construct normalization factors. As observed in Fig. 4 opposite orientations for the intergroup variations were chosen as recommended. For normalization the geometrical mean of 18S and *EF1* was used and calculated for each

biological replicate. Relative gene expression was calculated with the ΔΔCt method including the PCR efficiencies of the target and reference gene according to Pfaffl (Pfaffl, 2001).

The mathematical model to determine the relative quantification of target genes as follows*: sert, 5-ht$_{3a}$ and 5-ht$_{3b}$, dopamine d$_2$ and d$_3$, mao and vmat* mRNA expression in brain of *D.labrax* after FLUX and VEN exposures in comparison to the selected reference genes was performed according to Pfaffl (Pfaffl, 2001). Real-time RT-PCR allows quantification of rate transcripts and changes in gene expression (Pfaffl, 2001). Relative quantification is based on the expression levels of a target gene versus a reference gene. To calculate the expression of a target gene in relation to an adequate reference gene a mathematical model was established, although several mathematical models have been developed. This calculation is based on the comparison of the threshold values (*Ct*) at a constant level of fluorescence (Pfaffl, 2004). After we obtained the values of *Ct* of target and reference samples was calculated the relative expression ratio I based on *E* and *Ct* of target samples versus a control, and expressed in comparison to a reference gene. This model is based with efficiency correction. The model used as follows:

$$ratio\ (R) = \frac{(Etarget)^{\Delta CT(control-sample)}}{(Ereference)^{\Delta CT(control-sample)}}$$

Equation 1. Formula used in the calculation of the relative quantification of a target genes in comparison to a reference gene

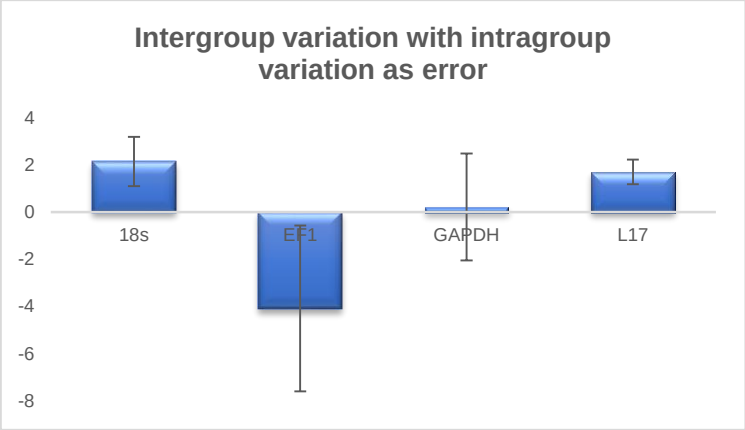


Fig. 4. Multiple reference gene normalization: *18s, EF1, L17 and GAPDH* of FLUX and VEN were analyzed with the software program Normfinder.

2.2.11. Statistical data analysis

Treatment effects were evaluated by means of a two-way ANOVA, at a 5% significance level. Before our hypotheses being evaluated by two-way ANOVA, two tests may be approved to proceed with the evaluation: (i) normality test, (ii) equal variations. Some data had to be square root or rank statistical transformed in order to fit ANOVA assumptions. All tests were performed using the software Sigma plot 11.1 version.

2.2.12. Ethics statement

This study has been carried out in accordance with the national regulations on animal experimentation as detailed in the Portuguese Animals and Welfare Law (Decreto-Lei nº 197/96 – http://dre.tretas.org/dre/78012/) approved by the Portuguese Parliament in 1996. Animal experiments were approved by the Institutional ethics committee of CIIMAR. During the whole experiment, fish were humanely treated. Before being sacrificed, all fish were anesthetized using tricaine methanesulphonate (MS222, 100% W/W Powder for solution for fish treatment – Pharmaq MS-222®), and every effort was made to minimize suffering.

Table 2. Used primers for target genes (and accession numbers for corresponding sequences)

Target gene (accession number)	Primer sequence (5'→3')		Amplicon l	Product (bp)	Efficiency of the PCR reaction	R^2 of the PCR reaction	Range Ct (cycle) of the PCR reaction	Homology with other species	Annealing temperature
SERT (002060)	F	GTTGATGGCAGTGTTTGGTG	20bp	109	92.0%	93.0 %	28-35	*Stegastes partitus* – 96%; *Takifugu rubripes*– 93%; *Danio rerio* – 88%	56 ºC
	R	GAAGATGGGGCAGATGTGTT	20bp						
$5HT_{3B}$ (00046560)	F	TCATCTGGCTGAATGTGTGC	20bp	91	100 %	99.1 %	6-24	*Larimichthys 25rócea* – 91%; T*akifugu rubripes* – 83%; *Homo sapiens* – 49%	
	R	AACTCCGCAGTCGTATTTCC	20bp						
$5HT_{3A}$ (00148200)	F	TGTATGTGGTGAACCTGCTG	20bp	186	96.0 %	99.8 %	9-25	*Stegastes partitus* – 75%; *Poecilia Formosa* – 65%; *Danio rerio* – 64%	
	R	TGTGTATCCCAGAATGAGGG	20bp						
Dopamine D_2 (00129960)	F	GGGTTGTTTTCAGGACCAAG	20bp	121	107.0%	96.9 %	29-35	*Larimichthys crocea* – 88%; *Notothenia coriiceps* – 79%	
	R	CGCAGAAAGGAGAAGAGCAA	20bp						
Dopamine D_3 (00076420)	F	GAATGAGCTGCGAGGTGAA	19bp	80	92 %	96.6 %	28-34	*Larimichthys 25rócea* – 96%; *Poecilia Formosa* – 91%; *Danio rerio* – 76%	
	R	CGTGGGTTGTTGGTATGAGA	20bp						
MAO (00054530)	F	GCCAATCACCTCAACCAAAC	20bp	100	104.0 %	99.0 %	26-32	*Larimichthys 25rócea* – 85%; T*akifugu rubripes* – 72%; *Danio rerio* – 62%;	
	R	AGGGACAAACCAAACACTGG	20bp						
VMAT (00018080)	F	CACCAAGAAGCTGCACAATG	20bp	86	104.0 %	90.0 %	26-33	*Oryzias latipes* – 90%; *Poecilia Formosa* – 89%; *Danio rerio – 81%*	
	R	TGAAGGGGTTGGTTATCAGC	20bp						

* F-Foward; R-Reverse

2.3. RESULTS

2.3.1. Identification of homology of the target sequences with others species

Based on the degrees of homology with the same genes in mammals and other fish species, we were able to confirm the identity of the partial gene sequences for *sert, 5-ht$_{3a}$ and 5-ht$_{3b}$* serotonin receptors, *d$_2$* and *d$_3$* dopamine receptors, *mao* and *vmat* in *D. labrax*. For the aim of sequence identification, we applied (i) blastx alignments, (ii) Clustal W alignments, and (iii) phylogenetic analyses.

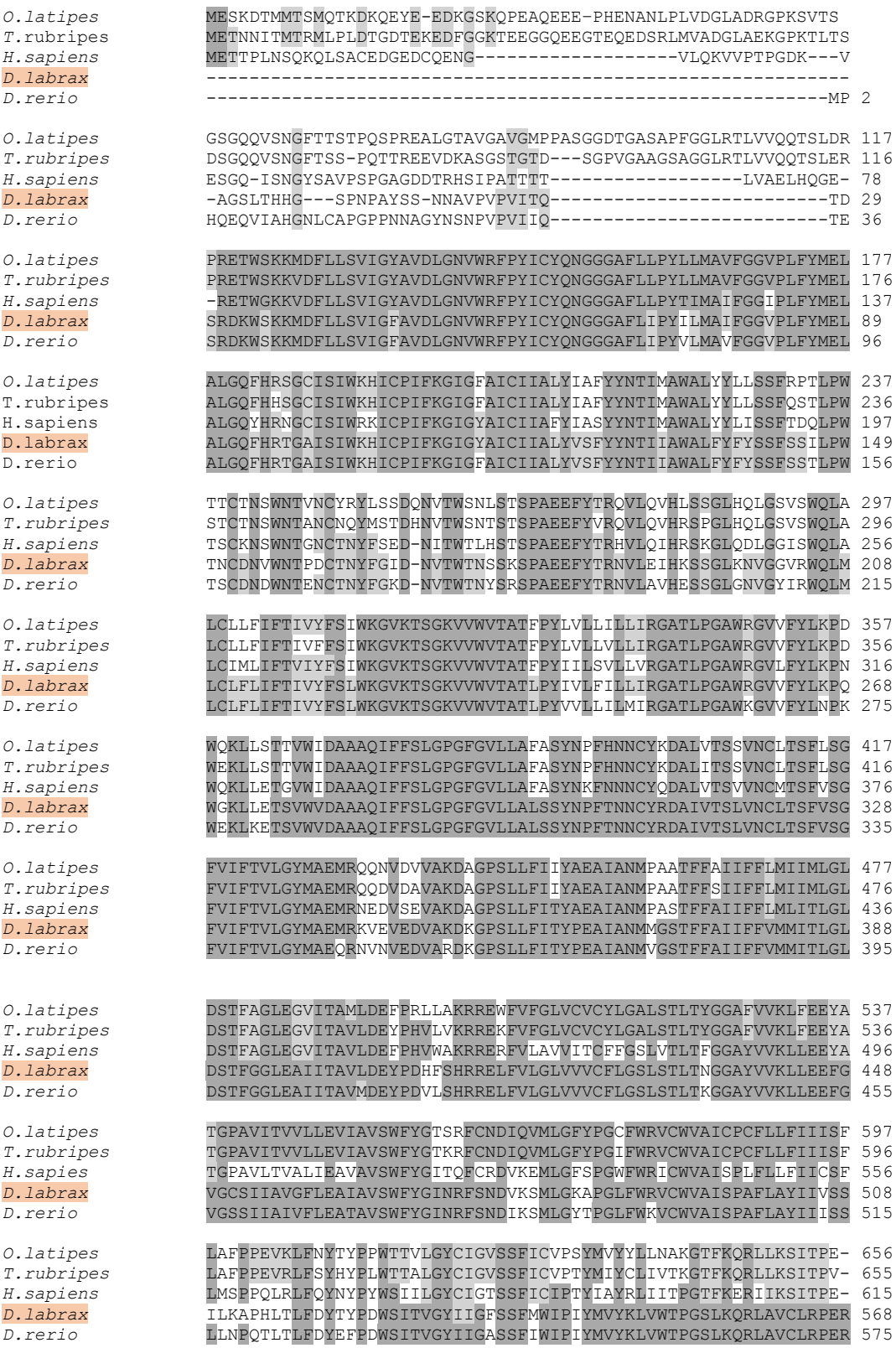
First, *blastx* alignments were performed, which showed high degrees of homology of the identified transcripts with other fish and mammal species, including human (Table 2). It is noteworthy that was recently identified by Tine et al., (2014) by phylogenetic tree, showing the relationships between *D. labrax* and other teleost fish species.

Secondly, EMBL-EBI Clustal W2 alignments (http://www.ebi.ac.uk/Tools/msa/clustalw2/) were performed, which showed high degrees of homology of the identified transcripts with other fish species, and human. Multiple alignments of the deduced amino acid (a.a) sequences in *D. labrax* with other fish, as *Oryzias latipes* (*O. latipes*), *Takifugu rubripes* (*T. rubripes*), *Homo sapiens* (*H. sapiens*) and *Danio rerio* (*D. rerio*), are shown in the figures below. In the partial amino acid sequences *of sert, 5-ht$_{3a}$ and b, d$_2$* and *d$_3$, mao* and *vmat* the highly conserved regions were marked in gray color. The amino acid sequences of *sert, 5-ht$_{3b}$, d$_3$* and *vmat* had a higher homology among selected species showing a high degree of sequence similarity and hence, conservation. In contrast, the amino acid sequences of the *5-ht$_{3a}$*, *d$_2$* like receptor and *mao* (Fig. 5) were very dissimilar among the chosen species.

Thirdly, results of the phylogenetic analyses revealed that sequences identified in *D. labrax* are evolutionarily closer to the same genes in other fish species, mainly *D. rerio* and *T.rubripes* than to mammals (Fig.6). Importantly, identified sequences of neurotransmitter genes and receptor subunits were characterized as similar to other species, since sequences always clustered together, with a certain distance to other subunits.

2.3.1.1. Multiple alignment

a. *sert* multiple alignment



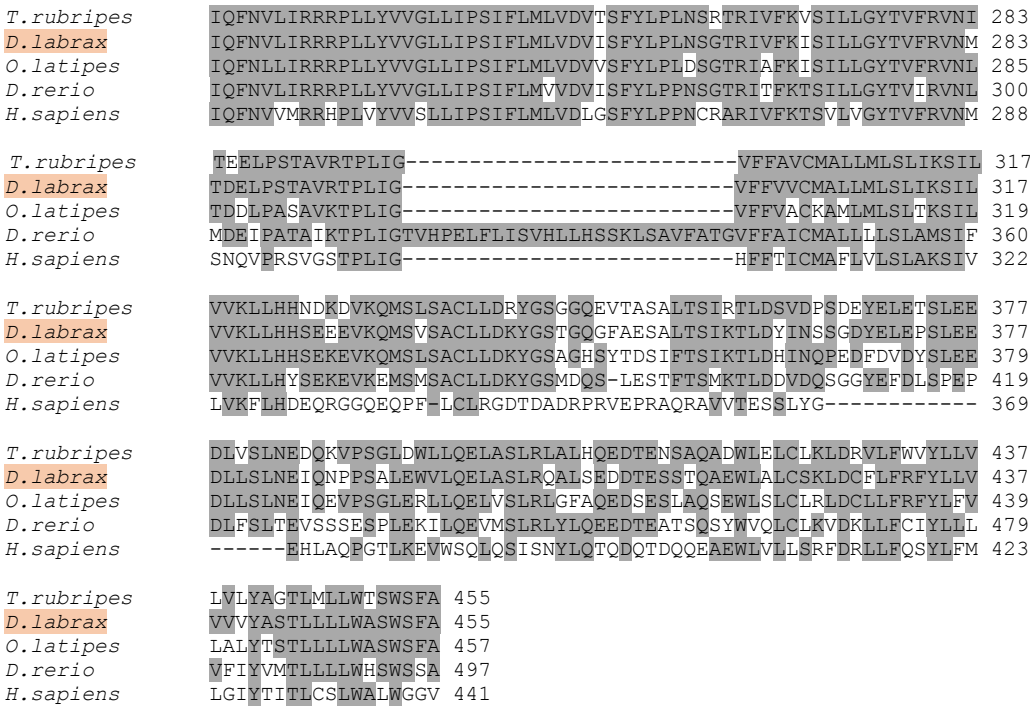
```
O.latipes          PSSENHRDYIVTNAI- 671
T.rubripes         PSTNQHRDIIVTSAL- 670
H.sapiens          TPTEIPCGDIRLNAV- 630
D.labrax           TIPDIHADSLNMATVP 584
D.rerio            TLPDIHTDGMSLSPVP 591
```

b. 5-ht$_{3a}$ receptor multiple alignment

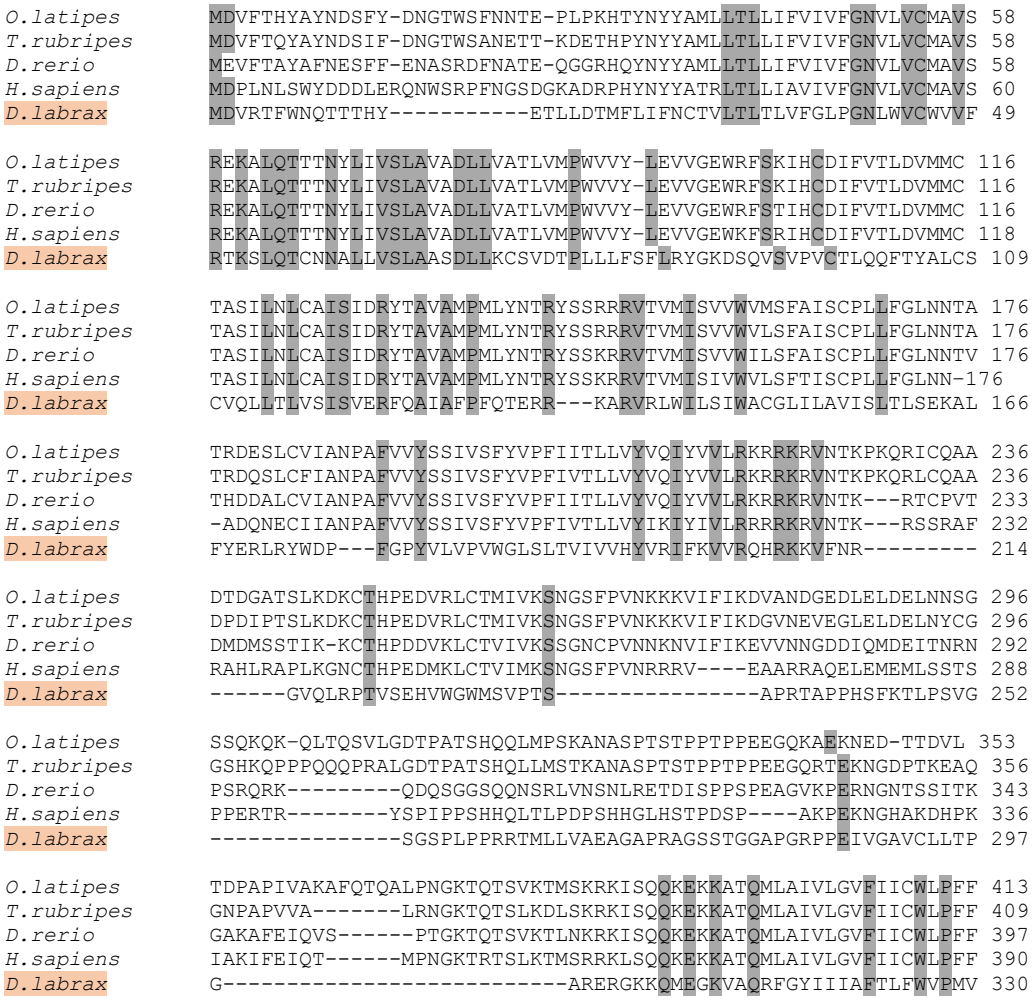
```
H.sapiens          ESSNYYAEMKFYVVIRRRPLFYVVSLLLPSIFLMVMDIVGFYLPPNSGERVSFKITLLLG 283
T.rubripes         DNDDYYAEMKFHVVIRRRPLFYTVNLLLPSIFLMVMDIVGFYLPPDSGERVSFKITLLLG 288
D.rerio            --------MYFWVLIRRRPLLYVVGLLIPSIFLMVVDVISFYLPPNSGTRITFKTSILLG 52
D.labrax           EDG----------QLRRRATLYVVNLLIPSCFLITVDLFSFLLPPQTVDRSSFKMTILLG 207
O.latipes          FGFN-QTVIVYTITMKRRSVLYIANFLLPVLFFLCLDFASFLMSDTGGEKVGFKVTVLLA 319

H.sapiens          YSVFLIIVSDTLPATAIGTPLIGVYFVVCMALLVISLAETIFIVRLVHKQDLQQPVPAWL 343
T.rubripes         YSVFLIIVSDTLPATAIGTPLIGVYFVVCMALLVISLTETVLIVRLVHKQDLQTPVPDWV 348
D.rerio            YTVIRVNLMDEIPATAIKTPLIGVFFAVCMALLLLSLAMSIFVVKLLHYSEKE------- 105
D.labrax           YTVFLLIMNDLLPIIGNTIPLINVFFSLCLALMVTSLLETIFITNLLSGSADFSPLPRWV 267
O.latipes          VTVMQLILNDILPPSSDKIPLIAIYCIGIFTLMLLSLLETILVMYLIDKDQKLNENP--- 376

H.sapiens          RHLVLERIAWLLCLREQS------TSQRP-----PATSQATKTDDCSAMGNHCSHMGGPQ 392
T.rubripes         KYVVLERAPVLFCIRRKHRLCSRLSSQGSDLDHYKDSSYGTSKDTQCTLHHTCEIGQRLS 408
D.rerio            ----VKEMSMSACLLDKY------GS--------MDQSLESTFTSMKTLDEVDQSGGYEF 147
D.labrax           RVFVLQFLGCLVCLPQKT-----------------KEPKGSGIKFSTAMAQRKADEGPPG 310
O.latipes          ------------------------------------EDKHSPLPCCCTEIKKWIRCASAAK 401

H.sapiens          DFEKSPRDRCSPPPPPREASLAVCG-LLQELSSIRQFLEKRDEIREVARDWLRVGSVLDK 451
T.rubripes         EHERESGLLRLGLPPRRDPTPPVMNNILQEVMAIRHFLEKRDRCREVAKEWLQVGYVLDV 468
D.rerio            DLSPEPDLFSLTEVSSSE---SPLEKILQEVMSLRLYLQEEDTEATSQSYWVQLCLKVDK 204
D.labrax           EMMPVAEGKAVQELRSLG----------RDLQAIRLQVEQQLGGSKSSEEWIQVGFIIDR 360
O.latipes          EDESFPKEGCSSPHTDVSLVMEKVYEELSEMKKSKYLLSSRTE-DEKPGYWTRVAEKINK 460

H.sapiens          LLFHIYLLAVLAYSITLVMLWSIWQYA 478
T.rubripes         LLFRVYLVAVVTYSITLGTLWSVWQVA 495
D.rerio            LLFCIYLLLVFIYVMTLLLLWHSWSSA 231
D.labrax           LLFGLYMVFISISFITIIIIWGNSYSQ 387
O.latipes          VFAVFYIIAVLLFLSVLFTVWNIDGT- 486
```

c. 5-ht$_{3b}$ receptor multiple alignment

```
T.rubripes         ---------------MSLIWLFIFF-SAHASQCVPEKPKRSALNQLTRTLLRQYDCGVR 43
D.labrax           ---------------MSLIWLTLLF-SAHLAECVPEKPKRSALNQLTRTLLRKYDCGVR 43
O.latipes          ---------------MSLIWMMLLFSVSAHLADCLPVKPKRSSLNQLTRTLLRNYDSGVR 45
D.rerio            MPRGTNAQVRVLGNVVRALGKLSLKCEVIPLAELVPEKPKRSALNQLTRLLLRKYNCGVR 60
H.sapiens          ----------MLSSVMAPLWACILV-AAGILATDTHHPQDSALYHLSKQLLQKYHKEVR 48

T.rubripes         PVHDWTSVTTVYIDLILLSVLDVDGKTQSITTSIWYRQVWTDEFLVWDPEEFDGLNEISL 103
D.labrax           PVYNWTSLTTVYIDLILQSVLDVDGNTQSITTSIWYRQIWTDEFLVWDPEEFDGINEISL 103
O.latipes          PVHNWTSTTTIYIDLILQSVLEVDGKTQSITTSIWCRQMWRDEFLVWDPEEFDGINEISL 105
D.rerio            PVQNWTKPTTIYIDLIIQAVLDVDGQTQKVTTSIWYRQIWNDEFLVWDPDEFDGITEISL 120
H.sapiens          PVYNWTKATTVYLDLFVHAILDVDAENQILKTSVWYQEVWNDEFLSWNSSMFDEIREISL 108

T.rubripes         SSDAIWIPDVIVTEFVDGGKSPPIPYIYVNSSGSVKNYRPMQVVLACSLEMYAFPFDKQN 163
D.labrax           SSDAIWVPDVIVSEFVDEGKSPTIPYVYVNSSGSVKNYRPMQVVVACSLEMYAFPFDKQN 163
O.latipes          SSDALWVPDVIVSEFVKEGKSPPIPYVYVNSSGSVKNYRPIQAVLACSLEMYAFPFDKQN 165
D.rerio            SSDAIWVPDLIISEFVDVGKSPVIPYVYVNCSGKVKNYKPIQVVSACHLEIYAFPFDKQN 180
H.sapiens          PLSAIWAPDIIINEFVDIERYPDLPYVYVNSSGTIENYKPIQVVSACSLETYAFPFDVQN 168

T.rubripes         CSLTFRSWLHSVKEIDLALLRSAEDIANDKREFMNDGEWELQSIPSHYWHIHQDATDYAQ 223
D.labrax           CSLTFRSWLHSVKEIDLALWRSAEAIANDKREFMNDGEWELLSIPSHYWIIHQDNTDYAH 223
O.latipes          CSLTFRSWLHSVNEIDLALWRSADAIANDKREFMNDGEWELLSIPSQYRQISQDNTDYSQ 225
D.rerio            CTLTFRSWLHSVSEVDLALWRPAEEIANDTREFMNDGEWELLGVPSRYWTLNLEDRDYAQ 240
H.sapiens          CSLTFKSILHTVEDVDLAFLRSPEDIQHDKKAFLNDSEWELLSVSSTYSILQSSAGGFAQ 228
```



d. Dopamine d_2 multiple alignment



```
O.latipes       ITHILNTH-CTKCKVPAEMYNAFTWLGYVNSAVNPIIYTTFNVEFRKAFIKILHC---- 467
T.rubripes      ITHILNTH-CTRCKVPAEMYNAFTWLGYVNSAVNPIIYTTFNVEFRKAFIKILHC---- 463
D.rerio         ITHIVN-----TYCQVPPELYTAFTWLGYVNSAVNPIIYTTFNIEFRKAFIKILHC---- 448
H.sapiens       ITHILNIH---CDCNIPPVLYSAFTWLGYVNSAVNPIIYTTFNIEFRKAFLKILHC---- 443
D.labrax        VILLMNVISWQNTDKLLMELETSAMVLTCVQAAVDPLIYTLVTRQFRSELSKILSSIPGC 390
```

e. *Dopamine d_3 multiple alignment*

```
T.rubripes      MAMFSSAEQPWNDSDGLSWAERNGSAEAPVQEE-RNYYAMLYSLLILAIVFGNVLVCLA 58
D.labrax        MALFSSAERLWNESDSLGWDDRNESSEASGQDE-RNYYAMLYSLLILAIVFGNVLVCLA 58
O.latipes       MAMFSSTEQLWNESEHLGWDERNDSSNKTGKDEGERNYYAMLYSLLILAIVFGNVLVCLA 60
D.rerio         MAMFSSGEWLWNDSE-HSFTPGGNYSPASGVEEAKRNYYAMLYSLLILAIVFGNVLVCIA 59
H.sapiens       MASLS------QLSSHLNYTCGAENSTGASQAR-PHAYYALSYCALILAIVFGNGLVCMA 53

T.rubripes      VLRERSLQTTTNYLVVSLAVADLLVASLVMPWAVYLEVVGGAWLFSRLYCNIFVTLDVMM 118
D.labrax        VLRERSLQTTTNYLVVSLAVADLLVASLVMPWAVYLEVVGGAWLFSRLYCNIFVTLDVMM 118
O.latipes       VLRERSLQTTTNYLVVSLAVADLLVASLVMPWAVYLEVVGGAWLFNRLYCNIFVTLDVMM 120
D.rerio         VLRERALQTTTNYLVVSLAVADLLVASLVMPWAVYLEVVGGAWLFSRLYCNVFVTLDVMM 119
H.sapiens       VLKERALQTTTNYLVVSLAVADLLVATLVMPWVVYLEVTGGVWNFSRICCDVFVTLDVMM 113

T.rubripes      CTASILNLCAISIDRYTAVVMPVLYN-TTHRSRKRVFVMIAIVWVLAFAVSCPLLFGFN 176
D.labrax        CTASILNLCAISIDRYTAVVMPVLYN-TTHRSRKRVFVMIATVWVLAFAVSCPLLFGFN 176
O.latipes       CTASILNLCAISIDRYTAVVMPVLYN-TTHCSRKRVFAMIATVWVLAFAVSCPLLFGFN 178
D.rerio         CTASILNLCAISIDRYTAVVMPVLYN-TTHSSRKRVSVMIATVWVLAFAVSCPLLFGFN 177
H.sapiens       CTASILNLCAISIDRYTAVVMPVHYQHGTGQSSCRRVALMITAVWVLAFAVSCPLLFGFN 173

T.rubripes      TTDDPMVCSISNPDFVIYSSVVSFYLPFIITLLVYIRIYIFLR-MRRKRIAFGQPSGKVQ 235
D.labrax        TTDDPMVCSISNPEFVIYSSVVSFYLPFIVTLLVYIRIYVFLR-MRRKRIAFGQASGKVQ 235
O.latipes       TTDDPMVCSISNPDFVIYSSVVSFYLPFIVTLLVYIRIYVFLR-MRRKRIAFGQARGKVQ 237
D.rerio         TTDDPAVCSISNPDFVIYSSVVSFYLPFAVTLLVYVRIYIFLR-RRRKKITFRQGSGKVQ 236
H.sapiens       TTGDPTVCSISNPDFVIYSSVVSFYLPFGVTVLVYARIYVVLKQRRRKRILTRQNS---Q 230

T.rubripes      PGSAPPSAETCLQ-ETPKAKQDLSPIRIKVQSVELPGPSKPSLLSGCLWRKRPKTGPVEN 294
D.labrax        PGSTPPSVETCLQEETPKAKQDLSPIRIKVQSVEPSGPSKPNLLSGCLWRKRPKTGPVEN 295
O.latipes       PGSTHPSVETCLQEETPKAKQDLSPIRIKVQSVESSGTSKPSLLSGCLWRKRPKTGPAQN 297
D.rerio         PASAPPSVETCLQDDAHKEKRDLSPIRINVITESKEQVIRPRLLANCLRRKRPQTAPAEN 296
H.sapiens       CNSVRP----------GFPQQTLSPDPAHLELKRYYSICQDTALGGPGFQERGGELKREE 280

T.rubripes      SMLPPVDTQNCCSISHASCGRTELDLEQERGEEGEEVAEGS-QREQPPVRMSCEVKDLSN 353
D.labrax        SMLPPVDTHNCCSISHASCGRTEFELEQER---AEEEAEGNNQHEQPPVRMSCEVKDLSN 352
O.latipes       SGLPPVDMQNYCSISHASCGRTEFDLEQGQ---VEQDASDSNQLELPPIRMNCEVKELSN 354
D.rerio         SLLPPVITLNYCSISQASFARTEQDANREE--------EEGGDEEQVAVRG-CEVKKLAN 347
H.sapiens       K------TRNSLSPTIAP------------------------------KLSLEVRKLSN 303

T.rubripes      GRTHTSLHPAYHSHTL----NTRFRTMHAREKKATQMLAIVLGVFLICWLPFFVTHILNT 409
D.labrax        GRTHTSLRPVPHSHTN----NPRFRSMHAREKKATQMLAIVLGVFLICWLPFFVTHILNT 408
O.latipes       GRTHTSLRPISHSQN-----NPRFRSMHAREKKATQMLAIVLGVFLICWLPFFVTHILNT 409
D.rerio         GRTHTSLRPPRAAHAMVCPSQARCRSMHSKEKKATQMLAIVLGVFLICWLPFFVTHILNT 407
H.sapiens       GRLSTSLKLG----------PLQPRGVPLREKKATQMVAIVLGAFIVCWLPFFLTHVLNT 353

T.rubripes      HCRTCYIPPGLYSAFTWLGYVNSALNPVIYTTFNIEFRRAFIKILSC 456
D.labrax        HCRTCYVPPGLYSAFTWLGYVNSALNPIIYTTFNIEFRRAFIKVLSC 455
O.latipes       HCRTCQIPPALYGAFTWLGYVNSALNPVIYTTFNIEFRRAFIKILSC 456
D.rerio         HCRACHIPPEVYSAFTWLGYVNSALNPVIYTTFNIEFRRAFIKILSC 454
H.sapiens       HCQTCHVSPELYSATTWLGYVNSALNPVIYTTFNIEFRKAFLKILSC 400
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f. *vmat multiple alignment*

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T.rubripes      MGKLDALWQLSLLRWLREERQSRKLILFIVFVALLLDNMLLTVVVPIIPSYLYSLDQS-58
D.labrax        MGRLDALRQFNLLKWLREERQSRKLILFIVFVALLLDNMLLTVVVPIIPSYLYNLDES-58
O.latipes       MGVLDALRQFRLIQWLREERQSRKLILFIVFVALLLDNMLLTVVVPIIPSYLYNLDES-58
H.sapiens       ----MALSELALVRWLQESRRSRKLILFIVFLALLLDNMLLTVVVPIIPSYLYSIKHEKN 56
D.rerio         MGLFDALRDFSLLTWLREERQSRRLILLIVFIALLLDNMLLTVVVPIIPSYLYTVDDE-58

T.rubripes      KVLVLKNSIESQEASSGAFQTIVSLYDHTVKTVGSNATSRPTELVPARLPGTELPGN-AT 117
D.labrax        TDVVLKNNTLSQHGPPGAFNSIVSLYDNTLRSSGSN---RSTDPVPSALAATELPQNSSS 115
O.latipes       ADVALRNDSLSQKGPEGSFHTIVSLYDNSIRLSSSNFTTRPADPVPPTLVVTKLQQNSSD 118
H.sapiens       ATEIQTARPVHTASISDSFQSIFSYYDNSTMVTGNATRDLTLHQTATQHMVTNASAVPSD 116
D.rerio         AAQMVKNHSMTPLSPSSTFQSIVSLYDNTTRVTGFSPQMSTAGPMSLAPTFVSPQNQ-SD 117
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T.rubripes      CPESTSMLTNENVKVGMMFASKATVQLITNPFIGPLTN---------------------- 155
D.labrax        CPQSTKKLHNENVKVGMLFASKATVQLITNPFIGPLTNRIGYQLPIFAGFCIMFLSTIMF 175
O.latipes       CPQSSSRLLNENVKVGMLFASKATVQLVTNPFIGPLTNRIGYQLPIFAGFCIMFLSTIMF 178
H.sapiens       CPSEDKDLLNENVQVGLLFASKATVQLITNPFIGLLTNRIGYPIPIFAGFCIMFVSTIMF 176
D.rerio         CPKADDQLLNENVKVGLLFASKATVQLITNPFIGPLTNRIGYQIPMFAGFCIMFVSTIMF 177

T.rubripes      ------------------------------------------------------------
D.labrax        AFSSSYALLFLARSLQGVGSSCSSVAGMGMLASVYTDDEERGHAIGIALGGLALGVLVGP 235
O.latipes       AFSSSYTLLFLARSLQGVGSSCSSVAGMGMLASVYTDDEERGHAIGIALGGLALGVLVGP 238
H.sapiens       AFSSSYAFLLIARSLQGIGSSCSSVAGMGMLASVYTDDEERGNVMGIALGGLAMGVLVGP 236
D.rerio         AFSSSYTLLFLARSLQGVGSSCSSVAGMGMLASVYTDDEERGNAIGIALGGLAMGVLVGP 237

T.rubripes      ------------------------------------------------------------
D.labrax        PFGSVMYDFVGKTAPFLILAFLAVFDGALQLFVLQPTKVEPESQKGTPLLTLMKDPYILI 295
O.latipes       PFGSVMYDFVGKTAPFLILAFLAVFDGALQLVVLQPTKVEPESQKGTPLLTLMKDPYILI 298
H.sapiens       PFGSVLYEFVGKTAPFLVLAALVLLDGAIQLFVLQPSRVQPESQKGTPLTTLLKDPYILI 296
D.rerio         PFGSVMYEFVGKTAPFLILAVLAVLDGALQLFVLQPSKVEPESQKGTSLITLMKDPYILI 297

T.rubripes      ------------------------------------------------------------
D.labrax        AAGAICFGNMAIAMLEPTLPIWMMETMCARKWQLGVAFLPASISYLIGTNIFGTLAHKMG 355
O.latipes       AAGAICFGNMAIAMMEPTLPIWMMETMCTRKWQLGIAFLPASISYLIGTNIFGTLAHKMG 358
H.sapiens       AAGSICFANMGIAMLEPALPIWMMETMCSRKWQLGVAFLPASISYLIGTNIFGILAHKMG 356
D.rerio         AAGSICFANMAIAMLEPALPIWMMETMCPRKWQLGIAFVPASISYLIGTNIFAVLAHKMG 357

T.rubripes      ------------------------------------------------------------
D.labrax        RWLCALIGIMVVGISIICVPFATDIYGLILPNFGVGFAIGMVDSSMMPIMGYLVDLRHVS 415
O.latipes       RWLCALIGMVLVGFSVICVPFAKDIYGLILPNFGVGFAIGMVDSSMMPIMGYLVDLRHIS 418
H.sapiens       RWLCALLGMIIVGVSILCIPFAKNIYGLIAPNFGVGFAIGMVDSSMMPIMGYLVDLRHVS 416
D.rerio         RWLCSLIGMLLVGISILCVPLAKDIYGLIVPNFGVGFAIGMVDSSMMPIMGYLVDLRHVS 417

T.rubripes      ------------------------------------------------------------
D.labrax        VYGSVYAIADVAFCMGFALGPSIGGSIAESIGFPWLMTIIGIVDIFFAPLCLFLRNPPGQ 475
O.latipes       VYGSVYAIADVAFCMGFALGPSIGGSIAENIGFPWLMTIIGIVDIFFAPLCIFLRNPPGQ 478
H.sapiens       VYGSVYAIADVAFCMGYAIGPSAGGAIAKAIGFPWLMTIIGIIDILFAPLCFFLRSPPAK 476
D.rerio         VYGSVYAIADVAFCMGFALGPSAGGAIARSIGFPWLMTIIGLVDIMFAPLCFFLRNPPAN 477

T.rubripes      ----------------------------------------
D.labrax        EEKIAILMDTNCSMKTRSYSTQGTYYQGENMDPEYDDYD 514
O.latipes       EEKIAILMDSNCSMKTRSYSAQGTYYQGESMDPEYDDYD 517
H.sapiens       EEKMAILMDHNCPIKTKMYTQNNIQSYPIGEDEESESD- 514
D.rerio         EEKMAILMDSNCSMKTRSYSTQ-GSSYQMGDDFDPESPE 515
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g. mao multiple alignment

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O.latipes       MVERRMSPVWKWVLVLFALASVILNIGLIVVHSGRTPRCAAQXXXXXLLAEHDARSGVF 60
T.rubripes      MVEQRMNSVIKWALILFVLVSIILNIVLISMYSGRAPKCSAHR-AHPLRGKHDERSLVF 58
H.sapiens       ------------------------------------------------------------
D.rerio         ------MTANAYDVIVIGGGISGLSAAKLLVDSGLNPVVLEARSRVGGRTYTVQNKETKW 54
D.labrax        --------MYECRVILSSSKALGVLEGSLGMEVTSTG------------PQTIQKAEGES 40

O.latipes       ADLSPEEYLQVQKYMLRQKDLD-ISTKQTTKPSENFLFLIDLLLPPKAEVLAFLDGNGPQ 119
T.rubripes      ADLTWEEYSQVQQYMLKQKDLD-ISTNQITKPSENFLFLIDLSLPKKADALAYLDDGKGK 117
H.sapiens       -----------GGKILKN-----VSVKRINASGN--------------RVLSVIADTE-28
D.rerio         VDLGGAYIGPTQNRILRIAKQYGVKTYKVNEEES---------------LVHYVKGKS-97
D.labrax        VTLGCSYTPSPSDSGELDIEWSVVSPDTTQKDQ---------------MLMSYTSGTK-83

O.latipes       PERRATVVVFHGSTGFIKEYVVGPLPTPTYHRDVTREKYGMELPLTKRTVTVGEYVLMFQ 179
T.rubripes      PTREATAVVFYGKSGYVKEYVVGPLPNPKYHRDVTKERYNTDIPINSRPVTIGEYAVLFE 177
H.sapiens       ----------NGEKEFTGDIFFSSMP----VKDLVGGISGIEIPETVKSAAAN------- 67
D.rerio         ----------YPFKGPFPPMWNPFAYMDYNNLWRTMDKMGMEIPKEAPWRAPH------- 140
D.labrax        ---------------YVHSNHALAKGLSFAARDPSMGDASLSISLLSPAHSAT------- 121

O.latipes       FFETEVFSKLQKVMAESFGVGPNRKLNAFEQMPRGVQSGDRKTWVSFFRDMSGMYIHPVG 239
T.rubripes      FLEAEFFSKLQKLMKESFDVDDTNHLNAFEQMPRGVRSGDRSTWISFMRDMSGMYIHPVG 237
H.sapiens       ------------------------------------------------------------
D.rerio         ------------------------------------------------------------
D.labrax        ------------------------------------------------------------


O.latipes       FEVLLNHQDLDPSRWSVERLLYNGVYFDSVEELNRAYDARSVERIVHKEAPDYGSLKPRP 299
T.rubripes      LEVLVNHESVNSSQWTIQRVLYNGQYFDSVQALKEKYDRGSVKKILYTKSRDYGSLKPKT 297
H.sapiens       ----LPYRDFVTVGLLVDRMLLK-----------NKTDIKTVNDIVPD------------ 100
D.rerio         ----AEEWDKMTMQQLFDKICWTRS--------ARRFATLFVNVNVTSEP---------- 178
D.labrax        ------------YQCKVKK--------------SPGVDMRKVSVVVMVKP---------- 145
```

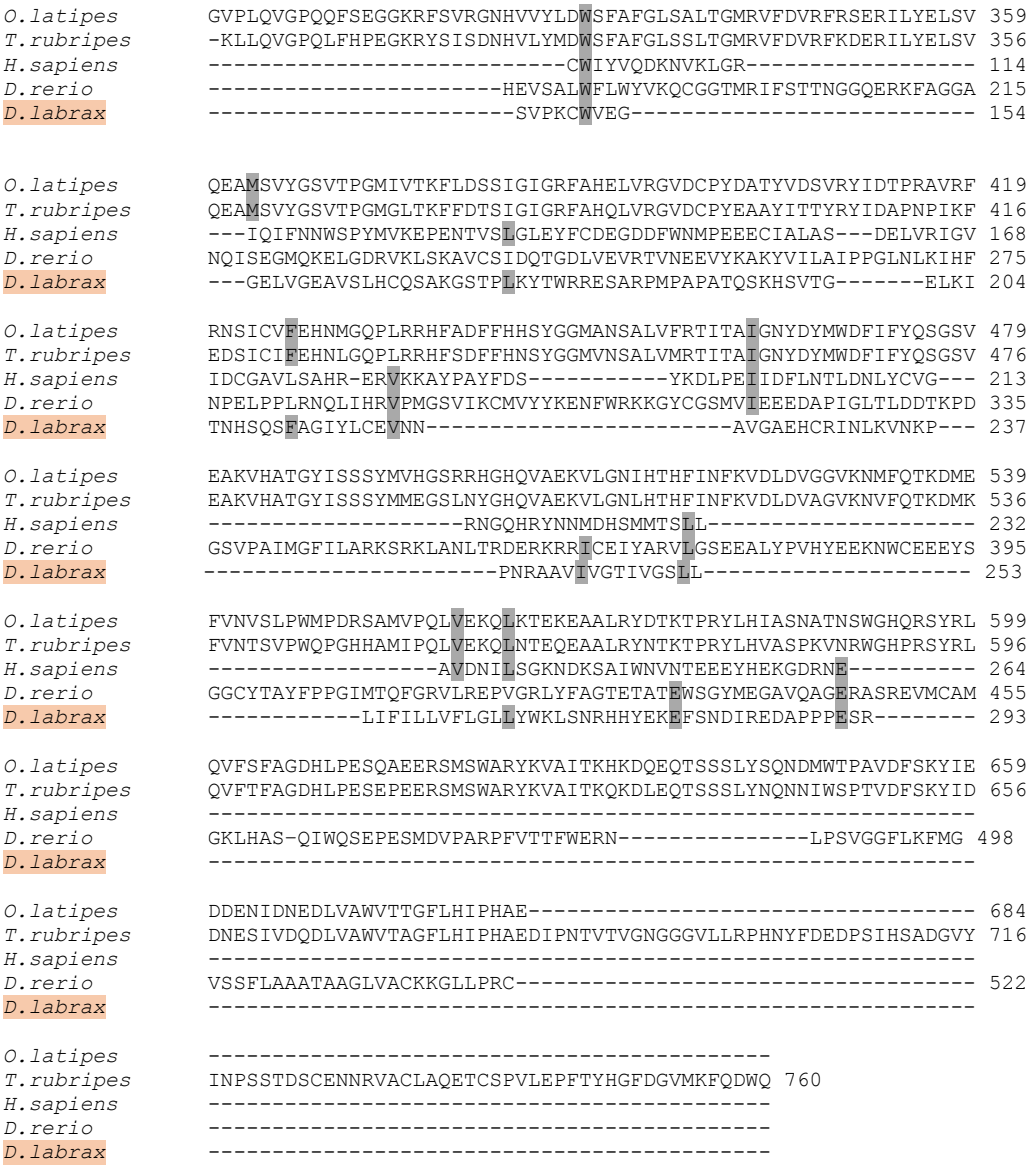
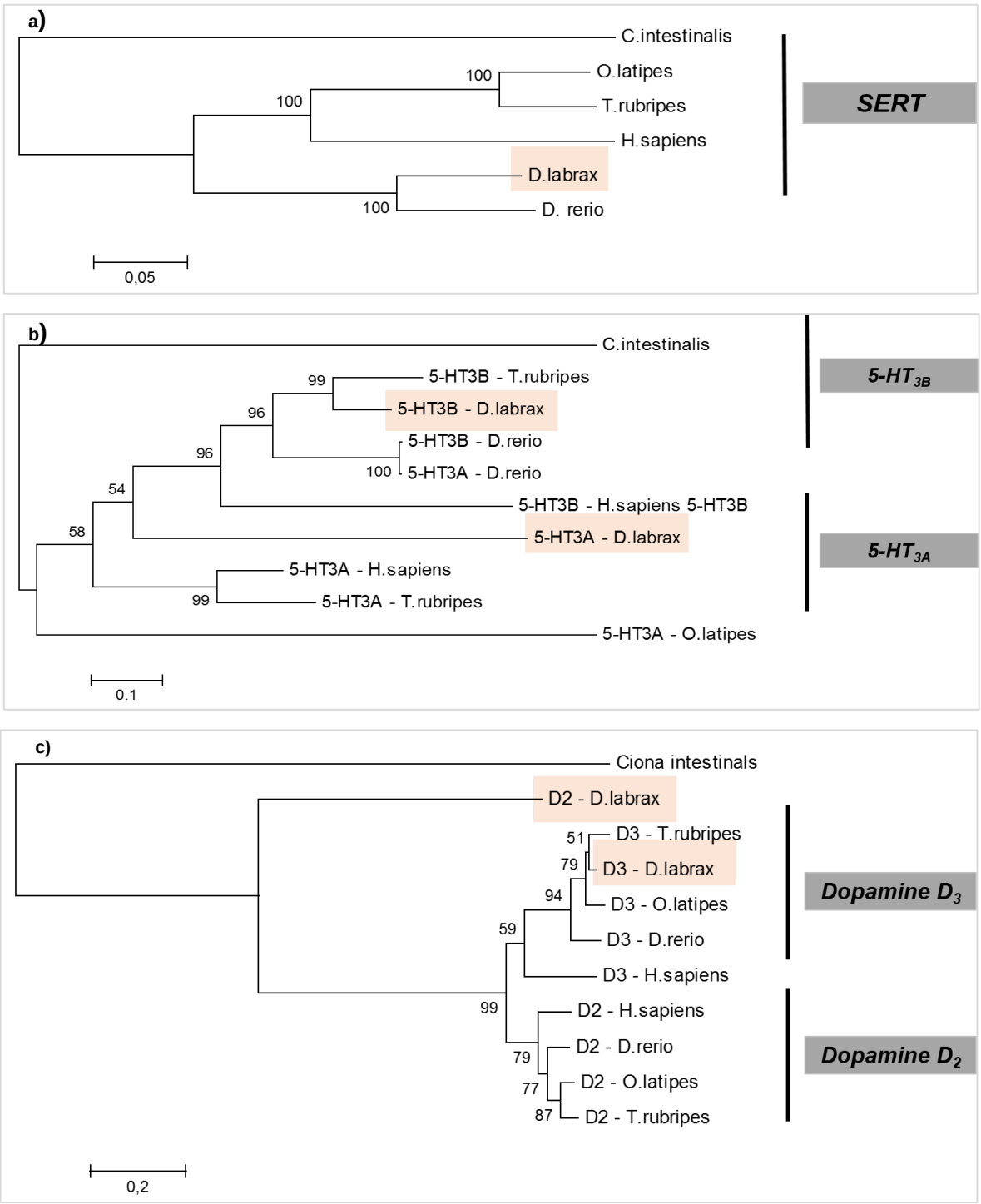


Fig. 5. Multiple alignments of the deduced amino acid sequences for *sert, 5ht$_{3a}$ and 5-ht$_{3b}$, dopamine dopamine d$_2$* and *d$_3$, mao* and *vmat* in *D. labrax* with other fish species and human using EMBL-EBI Clustal W2 alignments software.

SERT: D.labrax 002060; *H.sapiens*: EAW51223.1; *D.rerio*: NP_001170930.1; *T.rubripes*: XP_003968295.2; *O.latipes*:. XP_004075963.2. *5-HT$_{3A}$*: *D.labrax* 00148200; *H.sapiens* AAM21131.1*; D.rerio* XP_009296619.1; *T.rubripes* XP_011609568.1; *O.latipes* XP_011490786.1. *5-HT$_{3B}$*: D.labrax 00046560; *H.sapiens* NP_006019.1; *D.rerio* XP_009293724.1; *T.rubripes* XP_009293724; *O.latipes* XP_004076882.1. *D$_2$*: *D.labrax* 00129960; *H.sapiens* AAB26819.1; *D.rerio* NP_898891.1; *T.rubripes* XP_003968417.1; O.latipes XP_004075461.1. *D$_3$*: *D.labrax* 00076420; *H.sapiens* AAB08750.1; *D.rerio* AAH76352.1; *T.rubripes* XP_004081111.1; *O.latipes* XP_004081111.1. *VMAT*: *D.labrax* 00018080; *H.sapiens* NP_003045.2; *D.rerio* NP_001243154.1; *T.rubripes* XP_011618602.1; *O.latipes* XP_004077262.1. *MAO*: *D.abrax* 00054530; *H.sapiens* EKC75134.1; *D.rerio* AAO16681.3; *T.rubripes* XP_003964455.2 ; *O.latipes* XP_011477037.1.

2.3.1.2. Phylogenetic trees based on the multiple alignments



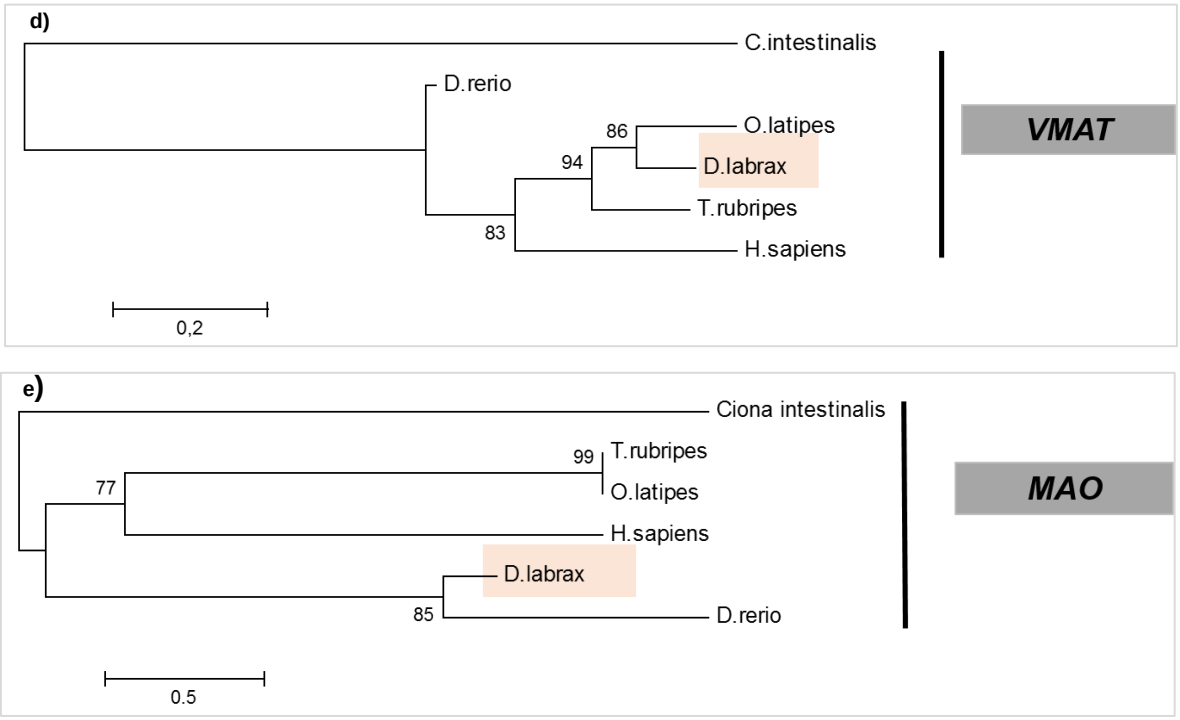


Fig. 6. Phylogenetic tree based on the multiple alignments (Clustal W) of related proteins from *sert, 5ht3a* and *b, dopamine d2* and *d3, mao* and *vmat* of *D. labrax* and others species. Evolutionary history was inferred using neighbor-joining (Mega 6 software) method and the percentage of concordance based on 1000 bootstrap iterations is shown at the nodes.

a)*SERT: D.labrax* 002060; *H.sapiens*: EAW51223.1; *D.rerio*: NP_001170930.1; *T.rubripes*: XP_003968295.2; *O.latipes*:. XP_004075963.2; *Ciona intestinalis*: XP_002125543.1. b) *5-HT$_{3A}$*: *D.labrax* 00148200; *H.sapiens* AAM21131.1*; D.rerio* XP_009296619.1; *T.rubripes* XP_011609568.1; *O.latipes* XP_011490786.1; *Ciona intestinalis*: XP_009862074.1 and b) *5-HT$_{3B}$*: *D.labrax* 00046560; *H.sapiens* NP_006019.1; *D.rerio* XP_009293724.1; *T.rubripes* XP_009293724; *O.latipes* XP_004076882.1; *Ciona intestinalis*: XP_009862074. c) *D$_2$*: *D.labrax* 00129960; *H.sapiens* AAB26819.1; *D.rerio* NP_898891.1; *T.rubripes* XP_003968417.1; *O.latipes* XP_004075461.1; *Ciona intestinalis*: XP_009860861.1 and c) *D$_3$*: *D.labrax* 00076420; *H.sapiens* AAB08750.1; *D.rerio* AAH76352.1; *T.rubripes* XP_004081111.1; *O.latipes* XP_004081111.1. d) *VMAT*: *D.labrax* 00018080; *H.sapiens* NP_003045.2; *D.rerio* NP_001243154.1; *T.rubripes* XP_011618602.1; *O.latipes* XP_004077262.1; *Ciona intestinalis*: XP_009860861.1 e) *MAO*: *D.abrax* 00054530; *H.sapiens* EKC75134.1; *D.rerio* AAO16681.3; *T.rubripes* XP_003964455.2 ; *O.latipes* XP_011477037.1; *Ciona intestinalis*: XP_002123175.1

2.3.2. Quantification of SERT, 5-HT$_{3A}$ and 5-HT$_{3B}$, dopamine D$_2$ and D$_3$, MAO and VMAT mRNA expression in brain of *D. labrax* after FLUX and VEN exposures

The effects of FLUX and VEN exposure in the mRNA expression of brain receptors transcripts was evaluated by qPCR-RT. Relative quantification was used for comparison of different mRNA expression of neurotransmitter genes and receptor subunits in the brain of *D.labrax.* Results are presented separately for VEN and FLUX exposure, first transporters of monoamines; second receptors of serotonin and dopamine; third and final the degrading enzyme.

2.3.2.1. Transporters of monoamines

2.3.2.1.1. Specific transporter of serotonin: *sert*

Transporter of monoamines, specific transporter, mRNA expression levels after VEN and FLUX exposure to different concentration are displayed in Fig.7 (A) and Fig.8 (B). No significant differences were observed for the mRNA expression of SERT in response to VEN exposure. In contrast, SERT mRNA was significantly increased in response to FLUX exposure at day 1 in the 50 µg/L treatment group compared to the solvent control.

A. RESPONSE TO VEN

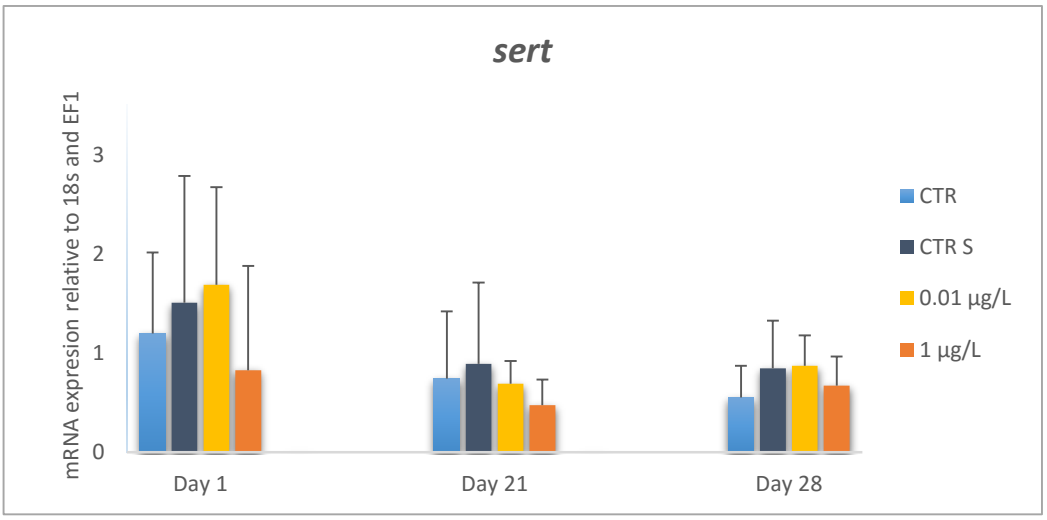


Fig. 7. Gene expression of the specific transporter of serotonin - *sert* - after exposure to two different concentrations (0.01 µg/L; 1 µg/L) of venlafaxine (A) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

B. RESPONSE TO FLUX

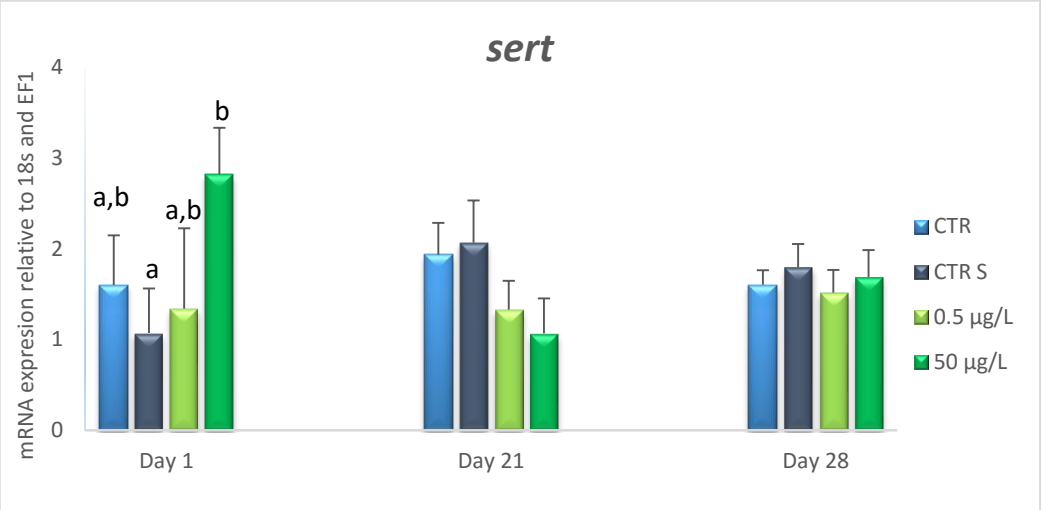


Fig. 8. Gene expression of the specific transporter of serotonin - *sert* - after exposure to two different concentrations (0.5 µg/L; 50 µg/L) of FLUX (B) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

2.3.2.2. Non-specific transporter: *vmat*

Non-specific transporter of monoamines, *vmat*, mRNA expression levels after VEN and FLUX exposure to different concentration are displayed in Fig.9 (A) and Fig.10 (B). A significant recovery was observed for the mRNA expression of *vmat* in response to VEN exposure at day 28 in 1 µg/L treatment group compared to the control. In contrast, *vmat* mRNA was significantly increased in response to FLUX exposure at day 1 in the 50 µg/L treatment group compared to the solvent control.

A. RESPONSE TO VEN

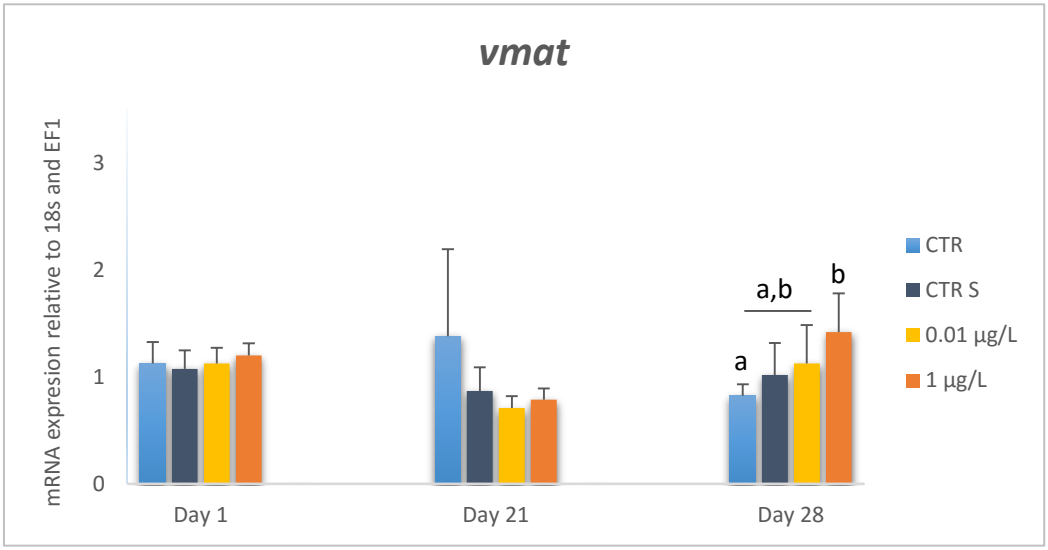


Fig. 9. Gene expression of the non-specific transporter of monoamines - *vmat* - after exposure to two different concentrations (0.01 µg/L; 1 µg/L) of VEN (A) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

B. RESPONSE TO FLUX

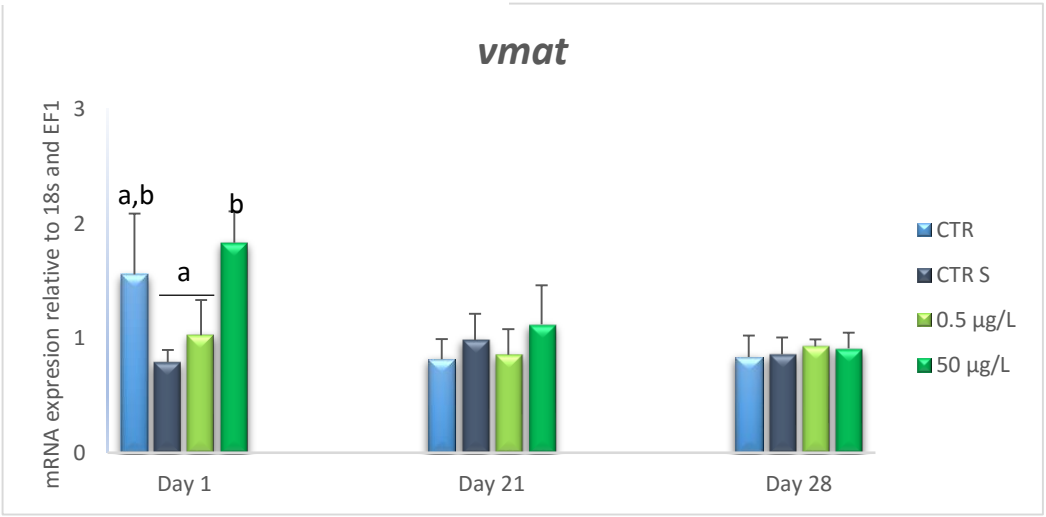


Fig. 10. Gene expression of the non-specific transporter of monoamines - *vmat* - after exposure to two different concentrations (0.5 µg/L; 50 µg/L) of FLUX (B) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

2.3.2.3. Receptors of serotonin

2.3.2.3.1. *5-ht$_{3a}$* receptor

Ion channel receptor of serotonin subtype *5-ht$_{3a}$* mRNA expression levels after VEN and FLUX exposure to different concentration are displayed in Fig.11 (A) and Fig.12 (B). Significant differences was observed for the mRNA expression of *5-ht$_{3a}$* in response to VEN exposure at day 21 in 1 µg/L treatment group compared to the solvent control. Although, *5-ht$_{3a}$* mRNA was significantly increased in response to FLUX exposure at day 1 in the 50 µg/L treatment group compared to the solvent control. In response to VEN observed chronic induction and in response to FLUX there was an acute induction in highest concentration.

A. RESPONSE TO VEN

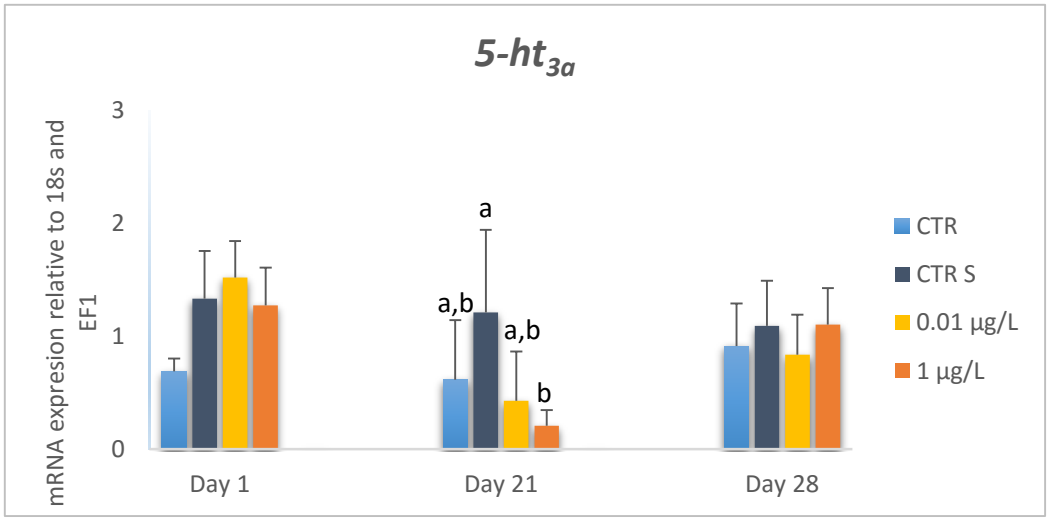


Fig. 11. Gene expression of receptor of serotonin subtype *5-ht$_{3a}$* after exposure to two different concentrations (0.01 µg/L; 1 µg/L) of VEN (A) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

B. RESPONSE TO FLUX

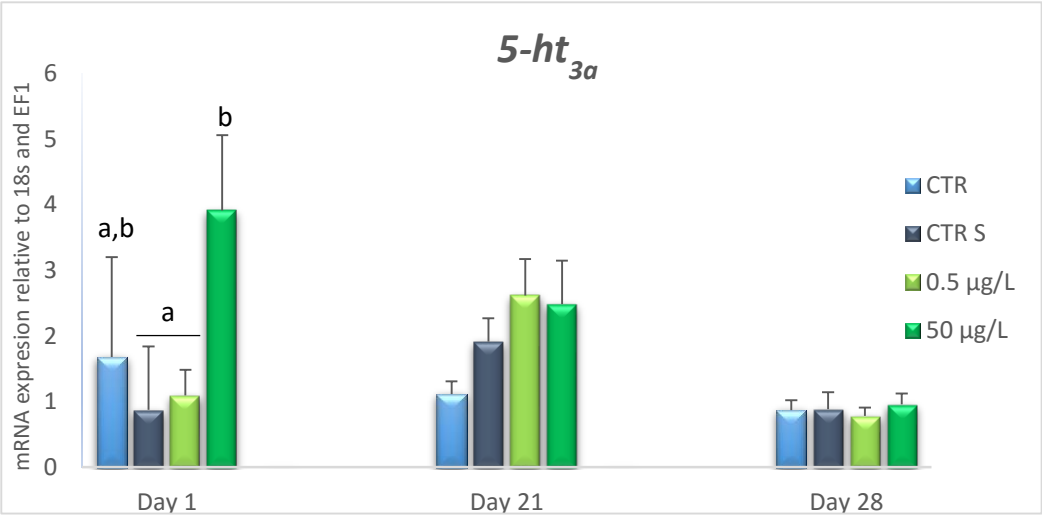


Fig. 12. Gene expression of receptor of serotonin subtype *5-ht*$_{3a}$ after exposure to two different concentrations (0.5 µg/L; 50 µg/L) of FLUX (B) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

2.3.2.4. *5-ht*$_{3b}$ receptor

Ion channel receptor of serotonin subtype *5-ht*$_{3b}$ mRNA expression levels after VEN and FLUX exposure to different concentration are displayed in Fig.13 (A) and Fig.14 (B). No significant differences were observed for the mRNA expression of 5-HT$_{3B}$ in response to VEN exposure in all exposure days. We observed in VEN exposure a tendency chronic at day 21, but without significant differences ($p<0.147$). In contrast, *5-ht*$_{3b}$ mRNA was significantly increased in response to FLUX exposure at day 1 in the 50 µg/L treatment group compared to the solvent control.

A. RESPONSE TO VEN

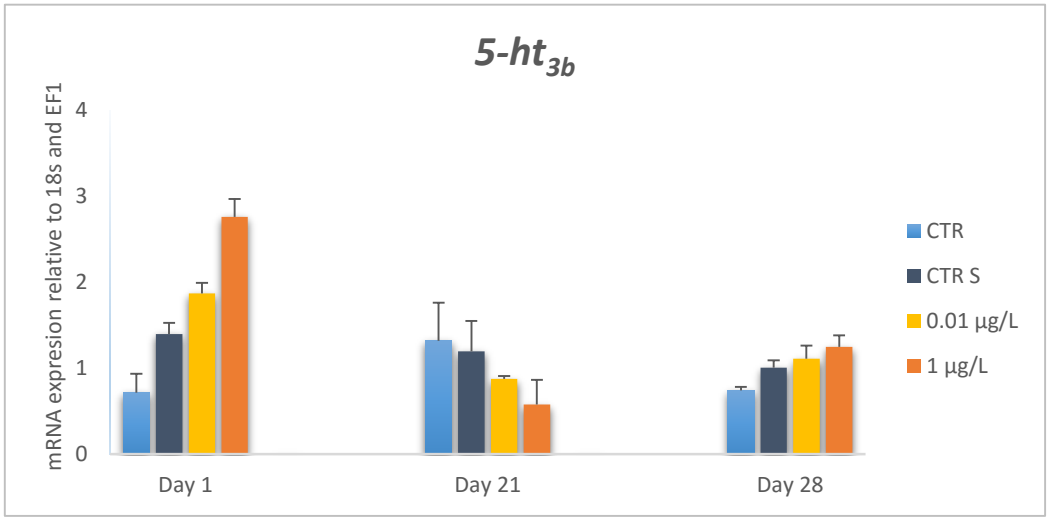


Fig. 13. Gene expression of receptor of serotonin subtype *5-ht*$_{3b}$ after exposure to two different concentrations (0.01 µg/L; 1 µg/L) of VEN (A) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

B. RESPONSE TO FLUX

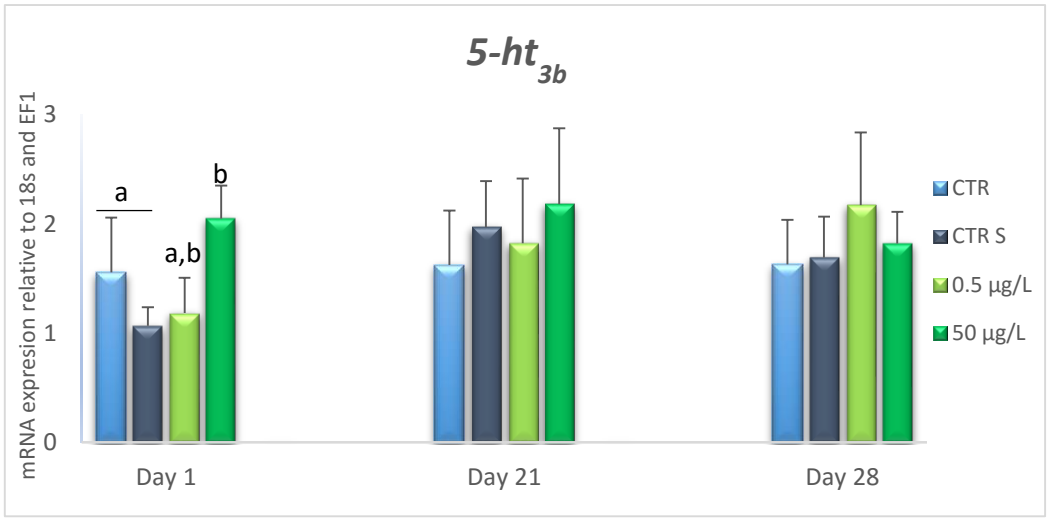


Fig. 14. Gene expression of receptor of serotonin subtype *5-ht*$_{3b}$ after exposure to two different concentrations (0.5 µg/L; 50 µg/L) of FLUX (B) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

2.3.2.5. Receptors of dopamine

2.3.2.5.1. Dopamine d_2 receptor

Receptor of dopamine subtype d_2 mRNA expression levels after VEN and FLUX exposure to different concentration are displayed in Fig.15 (A) and Fig.16 (B). A significant recovery was observed for the mRNA expression of dopamine d_2 in response to VEN exposure at day 28 in 1 µg/L treatment group compared to the solvent control. In contrast, dopamine d_2 mRNA was significantly increased in response to FLUX exposure at day 1 in the 50 µg/L treatment group compared to the solvent control.

A.RESPONSE TO VEN

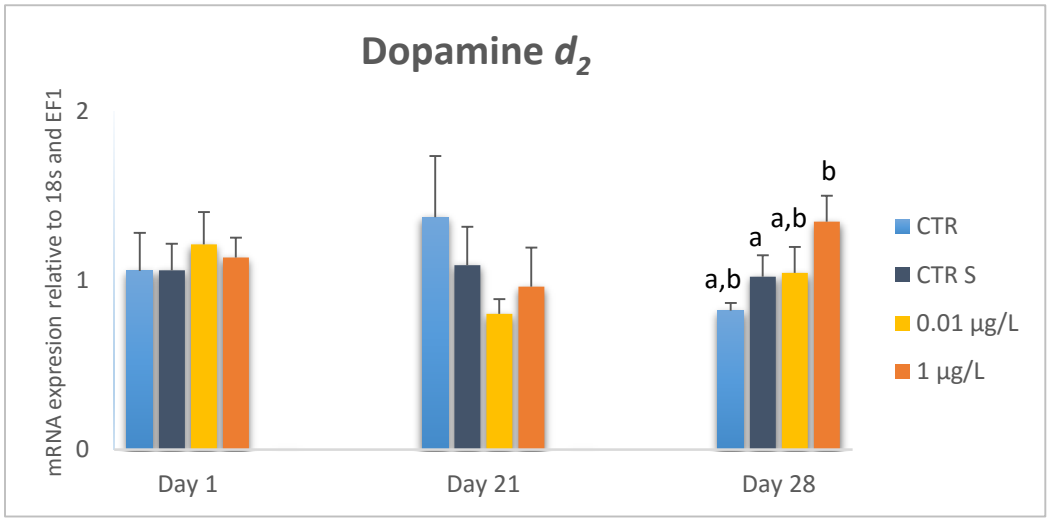


Fig. 15. Gene expression of receptor of dopamine subtype d_2 after exposure to two different concentrations (0.01 µg/L; 1 µg/L) of VEN (A) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

B.RESPONSE TO FLUX

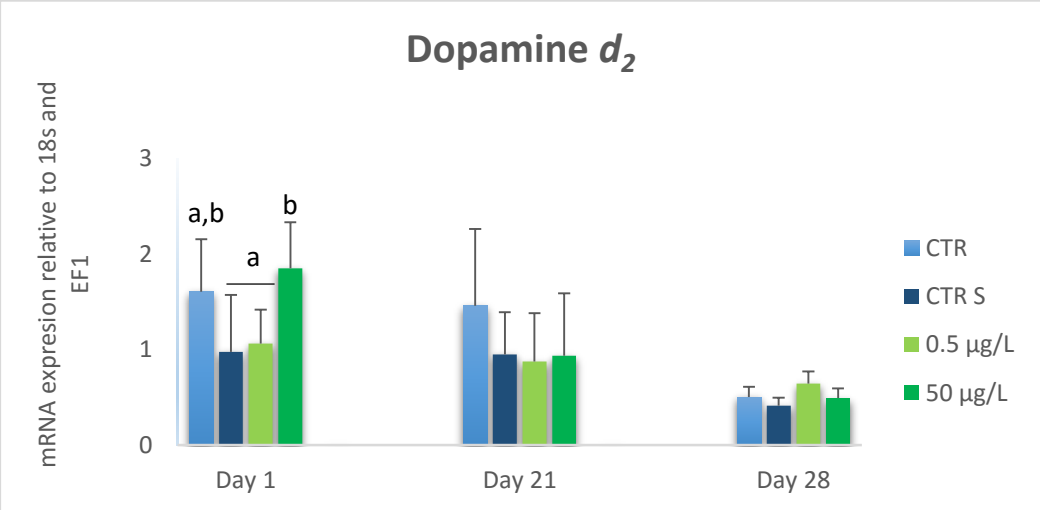


Fig. 16. Gene expression of receptor of dopamine subtype d_2 after exposure to two different concentrations (0.5 µg/L; 50 µg/L) of FLUX (B) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

2.3.2.5.2. Dopamine d_3 receptor

Receptor of dopamine subtype d_3 mRNA expression levels after VEN and FLUX exposure to different concentration are displayed in Fig.17 (A) and Fig.18 (B). A significant recovery was observed for the mRNA expression of dopamine D_3 in response to VEN exposure at highest concentration, 1 µg/L treatment group compared to the solvent control. In contrast, dopamine d_3 mRNA was significantly increased in response to FLUX exposure at day 1 in the 50 µg/L treatment group compared to the solvent control. Also, recovery was observed for the mRNA expression of dopamine d_3 in response to FLUX exposure at lower concentration, 0.5 µg/L.

A.RESPONSE TO VEN

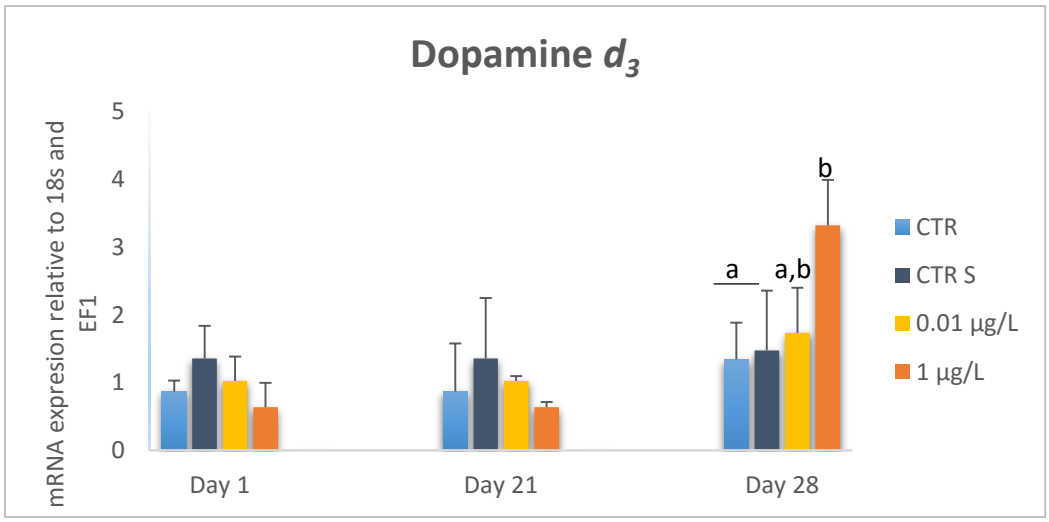


Fig. 17. Gene expression of receptor of dopamine subtype d_3 after exposure to two different concentrations (0.01 µg/L; 1 µg/L) of VEN (A) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

B.RESPONSE TO FLUX

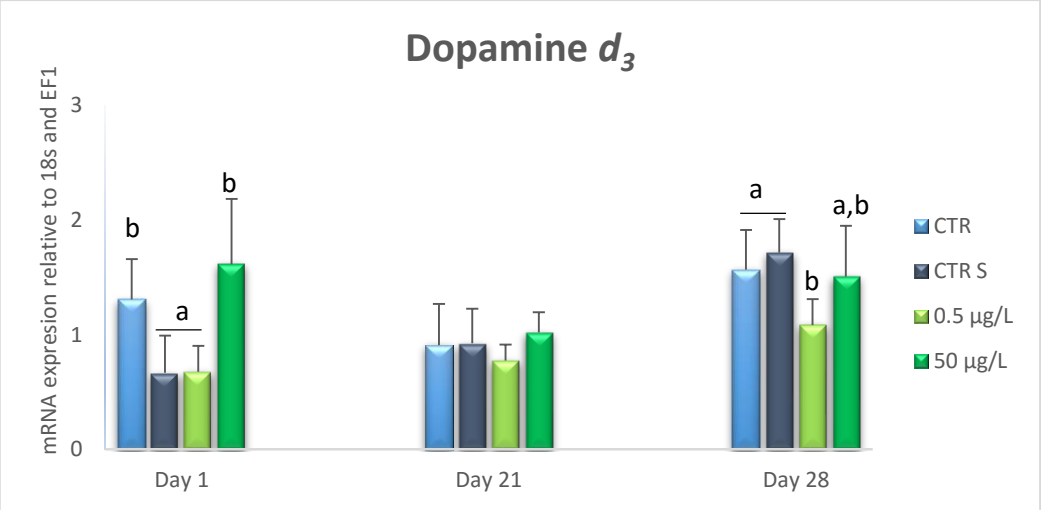


Fig. 18. Gene expression of receptor of dopamine subtype d_3 after exposure to two different concentrations (0.5 µg/L; 50 µg/L) of FLUX (B) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

2.3.2.6. Degrading enzyme: *mao*

Degrading enzyme mRNA expression levels after VEN and FLUX exposure to different concentration are displayed in Fig.19 (A) and Fig.20 (B). A significant recovery was observed for the mRNA expression of *mao* in response to VEN exposure at highest concentration, 50 µg/L, compared to the control group. In contrast, *mao* mRNA was significantly increased in response to FLUX exposure at day 1 and day 21 in the 50 µg/L treatment group compared to the solvent control. There were chronic and acute inductions to response FLUX.

The gene expression data of the target brain receptors show that there is an individual pattern of mRNA expression under pressure from the different two contaminants. Both responses are different each target gene.

A.RESPONSE TO VEN

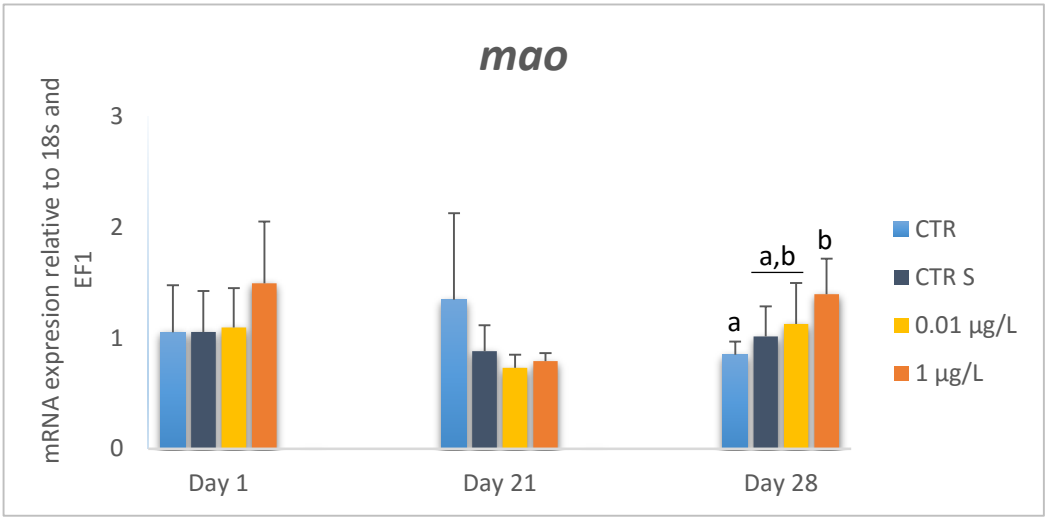


Fig. 19. Gene expression enzyme *mao* after exposure to two different concentrations (0.01 µg/L; 1 µg/L) of VEN (A) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

B.RESPONSE TO FLUX

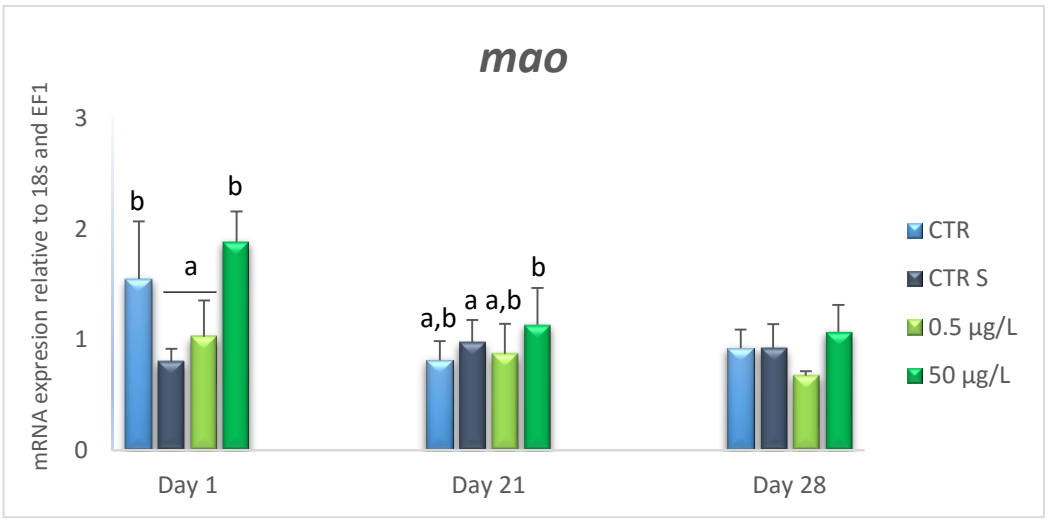


Fig. 20. Gene expression of enzyme *mao* after exposure to two different concentrations (0.5 µg/L; 50 µg/L) of FLUX (B) for 21 days and 7 days of recovery period. Differences in genes expression were analyzed between treatments and the solvent control (CTR S). Different letters denote significant differences at $p<0.05$.

2.4. DISCUSSION

The increasingly use of antidepressants by humans has resulted in their presence in the WWTP and ultimately in the aquatic ecosystems, posing an ever urgent problem for aquatic ecosystems worldwide (Schultz and Furlong, 2008; Vasskog et al., 2008; Kolpin et al., 2002; Metcalfe et al., 2003b; Gomez et al., 2006; Metcalfe et al., 2003a). Despite of the knowledge of environmental presence at low concentrations, the chronic effects of antidepressants on aquatics organisms are not well described or understood. Until now, no comparative studies on the ecotoxicity of SSRIs and SNRIs were performed in fish species, establishing whether they share a common mechanisms of toxicity since both share a common pharmacological activity (Brooks et al., 2003; Kreke and Dietrich, 2008; Owens et al., 1997). Overall, there is not enough studies to determine which PP are of concern (Brooks et al., 2003). Many studies focused on the freshwater fish species in behavioral alteration of PP on aquatic species, (Bisesi et al., 2014; Gaworecki and Klaine, 2008; Menningen, 2011; Thomas et al., 2012), locomotor activity or behavior, such as the ability to capture prey (Bisesi et al., 2014; Dufour-Rainfray et al., 2010; Gaworecki and Klaine, 2008; Larson and Summers, 2001; Lucki, 1998; Weinberger and Klaper, 2014), reproduction (Best et al., 2014; Foran et al., 2004; León-Olea et al., 2014; Prasad et al., 2015; Santos et al., 2010; Weinberger and Klaper, 2014) as well as appetite or feeding (Menningen, 2011).

It is very important to consider the potential effects of SSRIs and SNRIs on aquatic vertebrates at environmental concentrations and under chronic conditions, since the pharmacological targets are well conserved among species and pharmaceuticals are continuously release into the ecosystem. Brooks and colleagues (2003) suggested that chronic studies with environmental concentrations of pharmaceuticals are crucial and useful to assess aquatic ecosystem responses (Brooks et al., 2003). Neuroamine receptors may become desensitized by chronic exposure and allow further release of monoamines, allowing the therapeutic function of the chemical (Bisesi et al., 2014).

It is necessary to increase the knowledge about the responsse to chronic exposure in aquatic organisms and to compare two distinct PPs (SSRI and SNRI) on the neurotransmitter system to understand whether mRNA expression of the neurotransmitters system is altered when aquatic organisms are exposed to SSRIs and SNRIs and whether the pharmaceuticals classes of antidepressants have similar or different mechanisms of toxicity. Hence, the focus of this thesis was to understand how aquatic exposure to the antidepressant FLUX and VEN affect neurotransmission gene mRNA expression in the brain of juvenile European seabass and compare the chronic effects of SSRI and SNRI. Altered neurotransmitter gene expression may result from

antidepressants-induced epigenetic modifications, which could subsequently impact protein expression, synaptic plasticity and addictive behavior.

Also, we chose a marine fish species *D. labrax*, since few studies were performed elucidating the chronic response or mRNA expression after antidepressants exposure in marine species.

2.4.1. Homology of the target sequences with others species

The concern regarding an ecotoxicological impact of antidepressants in fish is based on the assumption that both the serotonergic system and dopaminergic neurotransmitter system is conserved between different vertebrate species including humans. Since both selected PP in this study, a SSRI (FLUX) and a SNRI (VEN), target the serotonin and dopamine neurotransmitter system, adverse effects can be expected. Our first analyses aimed to confirm the sequence identities of the available sequences of the neurotransmitter genes for European seabass, in particular of the available neurotransmitter receptor subunits, by comparing them to sequences of other vertebrate species including teleost fish.

Bioinformatic analyses performed on seabass sequences demonstrated that in general the amino acid sequences of the neurotransmitter genes and receptors of *D. labrax* are highly similar to other vertebrate species as *O.latipes, T. rubripes, H. sapiens*, and *D. rerio*. These results indicate a high degree of conservation in vertebrates between serotonin and dopamine receptors, their transporter and degrading enzyme.

Topology analysis revealed that partial amino acid sequences for the serotonin and dopamine receptors, transporters and degrading enzyme identified in this study are analogous. Moreover, the high degree of homologies with other fish and mammals seen after multiple alignments (Fig.5), and clustering of the sequences with other fish species in phylogenetic analysis (Fig.6) consequently demonstrate the presence of the genes in the brain of *D. labrax* and their sequence identity, which can be used to deduce a hypothetical function to the genes due to their sequence homology.

Concerning the dopamine receptors, only the subtype d_3 was similar with others selected species, while d_2 receptor was more dissimilar.

Sequence homology allows the assumption that *D. labrax* genes have similar functions as in other vertebrates. The potential functions of the genes targets are different. The receptors of serotonin *5-ht*$_{3a}$ and *5-ht*$_{3b}$, ligand-gated ion channel, are responsible for fast transmitting signals between neurons or release the monoamines (Chameau and Van Hooft, 2006). The receptors of dopamine, dopamine d_2 and d_3, promote signal transmission between neurons to different functions, as modulatory action to sensory

perception in the retina and olfactory bulb, regulation of prolactine release in the pituitary gland, control of body temperature, food intake and sexual behavior in the hypothalamus (Callier et al., 2003). The storage process of neurotransmitters is performed by *vmat* that is release at the synapse following neuronal membrane depolarization. When monoamines are not stored in presynaptic vesicles by *vmat,* they are degraded by *mao* enzyme (Maximino, 2012). *Sert* gene serves the important function of taking up serotonin release during serotonergic neurotransmission (Descarries and Riad, 2012). Thus, the main output of homology of the target sequences with others vertebrates is that the obtained sequences cluster and align well improving the available knowledge of similar/difference between species.

2.4.2. Neurotransmitters mRNA expression with FLUX and VEN exposure

Antidepressants are known to act on receptors of the neural system and at the level of neurotransmission. This indicates probable changes in target neural system and their mRNA expression, as we observed in this study. We were initially interested in FLUX and VEN because of four main reasons: they are highly prescribed drugs, they have a potential to interfere with behavior (Brooks et al., 2003) and possess physical-chemical properties that favor bioaccumulation (Weinberger and Klaper, 2014). These classes of antidepressants are relatively lipophilic and are prone to cross the blood-brain barrier and to interact with therapeutic targets (Bisesi et al., 2014; Brooks, 2014).

There was acute up regulation in all target genes, on 1st day of exposure of FLUX, except for *mao* that had chronic effects showing a pattern of increased expression at day 21. Two recent studies showed the FLUX can regulate the transcription of genes involved in biological pathways (Park et al., 2012; Thomas et al., 2012). FLUX and another SSRI, sertraline, can influence the transcription of many genes in zebrafish (Park et al., 2012). The larval zebrafish, 76 hours post fertilization (hpf), were exposed for 96h, to two SSRIs at two concentrations: 25 and 250 µg/L. They observed by microarray that the same five genes were differentially regulated in both SSRIs indicating shared molecular pathways, which were recognized to be involved in regulation of the stress response, as myogenin and FKBP5. Consistent with microarray results, FKBP5 gene expression was shown to be downregulated after exposure to the SSRIs and the pattern of gene expression changes was similar to that observed by microarray analysis. Most of the genes affected by FLUX were involved in transcription regulation and signal transduction, as SLC5A11 (solute carrier family 5 (sodium/inositol cotransporter) and SLC6A11 (solute carrier family 6 (neurotransmitter transporter), response to stress, as myogenin and FKBP5, among others. As in our study there were no changes in VEN exposure at 21 day of SERT

(SLC6A4) mRNA, in contrast with the exposure to FLUX in which there was a significantly acute induction at the first day in the highest concentration (50 µg/L). Consequently, in the same study they showed that no changes in expression of genes involved with reproductive functions were observed at low concentrations of both SSRIs, (Park et al., 2012). We know that *sert* is the target of SSRIs and SNRIs. Benmansour et al. (2002) show that SSRI-induced downregulation of the transporter. Interestingly, in 2000 was shown that low expression of *sert* in brain of mammal's results in abnormal serotonin transmission in the major component in brain (hippocampus) and was correlated with cognitive impairment, like Alzheimer's disease (Hu et al., 2000). Previous research showed that vertebrates, like aquatic species, when exposed to PP exhibit differences in behavior. Aggression frequently coincides with these emotionally differences in fish. Also, in rats, it was demonstrated that increased aggression are accompanied by an alteration in the expression of key genes that regulate system of serotonin neurotransmission included SERT in select brainstem nuclei (Kerman et al., 2011).

In our study, we demonstrated that serotonin receptors *5-ht$_{3a}$* was down regulated after VEN exposure at day 21. Although*, 5-ht$_{3b}$* mRNA had a tendency to inhibition at day 21, no significant differences were observed. A limited number of studies focused on the association of gene variants encoding the subtypes of serotonin receptor with the different responses of gene transcription due to exposure to antidepressants (O'Leary et al., 2014). Interestingly, the knockout of a subtype of these receptors, *5-ht$_{3a}$*, in mice resulted in resistance to FLUX exposure inducing anxiety (Smit-Rigter et al., 2012), suggesting a role for the *5-ht$_{3a}$* in the reported negative effects of SSRI treatment. Another study showed that FLUX can inhibit binding of a *5-ht$_3$* receptor antagonist in neuroblastoma-glioma hybrid cells (Lucchelli et al., 1995). The receptor *5-ht$_3$* is distributed throughout the brain and CNS, with relatively high concentrations in the spinal cord and brainstem (area postrema), where it regulates vomiting. The chronic treatment to FLUX leads to the desensitization of the *5-ht$_3$* receptor (Fan, 1994). It is noteworthy that, the SSRIs and SNRIs exerts its therapeutic effect by inhibiting monoamine transports, thus reducing the reuptake of the serotonin and activity of serotonin transporter protein (*sert*) at presynaptic neuronal membranes (Charoenphandhu et al., 2013; Descarries and Riad, 2012), which ultimately lead to an increase of serotonin concentration in the synaptic clefts. Down regulation in the expression of these genes, *5-ht$_{3a}$ and 5-ht$_{3b}$*, would reduce the sensitivity serotonin, and role in neurotransmission. Stimulation of 5-HT$_3$ receptor leads to ion influx via an ion channel, such as Na^+, K^+ efflux and Ca^+ influx, inducing a depolarization mediated by cation flow (Reiser, 1991). The major consequence of depolarization is a rapid increase in cytosolic Ca^{2+} concentration from the extracellular membrane followed by neurotransmitter release from peripheral and central neuron (Hoyer, 1994).

Importantly, the function of this receptor depends on its localization. The activation of postsynaptic neuron *5-ht*$_3$ receptors is involved in fast synaptic transmission, while nerve-terminal (presynaptic neuron) *5-ht*$_3$ receptors activation leads to release of neurotransmitters, like serotonin, dopamine or GABA. Thus, perturbed regulation of this particular genes is interesting because it has implications in (i) the regulation of synapses, for instance involved in fast synaptic transmission on postsynaptic neuron; (ii) the levels of neurotransmitters and their release of important neurotransmitters, as serotonin, dopamine, acetylcholine or GABA from presynaptic neurons to synaptic cleft. Release of these neurotransmitters in presynaptic neurons is crucial to active different receptors and modulates signals between neurons. In the experimental condition of PP exposure, which leads to an increase of monoamines in the synaptic cleft, the decreased receptor mRNA expression of 5-HT$_{3A}$ and *5-ht*$_{3b}$ may be involved in the counter-regulation of serotonin level or desensitization. Considering the complex nature of monoamine systems and the interplay with other neurochemical system, numerous mechanisms may play a role in down regulation. Currently, reduced excitatory inputs or excessive self-inhibition by others serotonin receptors, as *5-ht*$_{1a}$, reduce serotonin synthesis and/or tryptophan deficit, as seen in mammals (Artigas, 2012b). From literature we know that in mammals the depression is attributable, at least in part, to abnormal transmission at central serotonin synapses (Andrews, 2015). Summarizing, we can suggest that the observed down-regulation of *5-ht*$_{3a}$ *and 5-ht*$_{3b}$ receptor mRNA expression can have effects on the fitness of aquatic animals with impact on their physiology as the ability to alter monoamines levels or neurotransmitter systems can influence diverse physiological processes in fish including: control of locomotor activity, appetite, reprodutive fitness, sleepor agreesion (Bisesi et al., 2014; Brooks et al., 2003; Mennigen et al., 2011, 2010; Menningen, 2011).

Furthermore, the response to VEN antidepressant resulted in a unique and different gene expression profile. During the recovery period (without antidepressant exposure), the Dopamine D_2 and D_3 receptor, within D_2-like subtypes, mRNA increased significantly at day 28 VEN or FLUX exerts its therapeutic effect by inhibiting monoamine transports reducing the reuptake of the serotonin or dopamine and activity of serotonin or dopamine transporter protein (SERT or DAT) at presynaptic neuronal membranes. We hypothesize that during the exposure with VEN the monoamine levels in the synaptic cleft were increased; the lack of reuptake inhibition should then lead to a decrease of previously elevated dopamine level. The mRNA expression of both dopamine receptors (D_2; D_3) was negatively correlated to the hypothesized change of dopamine level in the synaptic clefts, and may be related to an auto regulation to maintain high level of dopamine neurotransmission. It was reported that the presynaptic dopamine receptor, most of which belongs to the class D_2-like, act as auto receptors (Vallone et al., 2000). These self-

receptors recognize the density of dopamine from the synapse and reduce dopaminergic tone, decreasing the synthesis of dopamine in the presynaptic neuron and reducing neuronal discharge rate and the release of this monoamine (Harley, 2004).

D_2 receptor is thought to act predominantly as an auto receptor, and lower levels of expression may result in enhanced dopamine signaling (Sun et al., 2015). This study demonstrated that amphetamine self-administration decreased D_2 receptor and increased D_3 receptor mRNA level in ventral tegmental area or only increased the mRNA level of D_3 receptors in the nucleus accumbens without any effect on either D_2 receptors.

A previous study exposed Hybrid striped bass to VEN at concentrations of 50, 250, and 500 µg/L with a 6 day recovery period. At day 3 there was a significant increase of serotonin concentrations on brain that remained elevated for all treatments after 6 days of depuration (Bisesi et al., 2014) and could be related to inhibition of serotonin receptors. Studies had shown that dopamine plays a major role in aggression and social dominance. Following aggressive social interactions dopamine was shown to be decreased in the hippocampus of rainbow trout while increasing in the hypothalamus (Carpenter et al., 2009).

It is essential to regulate the neurotransmitters levels and theirs receptors in brain of organism, because the neurotransmitters play an important role in organism fitness. So, in our study was crucial understand if in response to VEN there were significant recovery in dopamine D_2 and D_3 after depuration time, or significant increase in response to FLUX at first day of exposure, leading to regular neurotransmission signals in European seabass that could alter the fitness of the species.

The present study showed for the first time the comparison of mRNA expression of neurotransmitter genes and receptor subunits in response to the exposure to a SSRI and a SNRI in the brain of a marine fish species, *D. labrax*. As we have observed in the gene expression pattern, both pharmaceutical have unique and differently profile. The effects of VEN appeared after 21 days of exposure and had specific effects on serotonin and dopamine receptor subunits. In contrast, the effects of FLUX appeared only at day 1 of exposure, and affected all analyzed neurotransmitter genes and receptors.

2.5. CONCLUSION

The present work aimed to identify changes on neurotransmitters gene and receptors of serotonin or dopamine changes in transcription levels that could be useful for the considerations of potential effects in fish species fitness.

The multiple alignment of the amino acid sequences for *sert, 5-ht$_{3a}$ and 5-ht$_{3b}$ receptors, dopamine d$_2$ and d$_3$ receptors, mao* and *vmat* in *D. labrax* with other vertebrate

species including human showed high degrees of homology of the identified transcripts. The observed sequence similarity allows the speculation that compounds could affect similar targets in non-model species, as we analyzed in the marine fish species *D. labrax*. The results on gene expression can indicate that environmental exposure to antidepressants may adversely affect aquatic species triggering behavioral disorders and aggravating the fitness. Furthermore, the sequence homology and the wide geographical distribution range make European Sea bass an interesting candidate as a sentinel species for environmental field studies.

The antidepressants share a common pharmacological activity, despite in our results they do not share common effects on analyzed neurotransmitter genes and receptors. First, the response to VEN was specific and selected to some target genes, since significant effects of antidepressants were observed in several target genes. It was detected a chronic induction mainly of the serotonin *receptor 5-ht$_{3a}$* and *5-ht$_{3b}$* at 21 day. Regarding dopamine receptors, *d$_2$* and *d$_3$* were significantly recovered in time of depuration. In contrast with FLUX, all neurotransmitter genes and receptors were significantly regulated by the contaminant at first day of exposure, leading to an acute induction of gene expression. The presented data indicate that both antidepressants have different molecular mechanisms in a non-target species.

The *D. labrax* was used for the first time as a representative of a marine fish species for the analysis of chronic effects of PP on the neurotransmitter system. This is particularity true because until nowadays only research in freshwater fish was present dealing with effects of PP. But, an increasing number of scientists are taking advantage of marine organisms as models to investigate questions in biology. The chosen marine species, *D. labrax*, is an economically important species of fish, which increases the relevance of this study. Observed neuronal changes could have major impact on European seabass, and may finally lead to alterations on their fitness.

Hence, the qPCR-RT analysis revealed the potency of SSRI and SNRI in modulating expression of genes involved in neurotransmission response pathways. Changes in the expression of the target gene were useful as biomarker of effects of chronic exposure to SSRI or SNRI. Data indicate the need to test pharmaceutical compounds individually, in order to provide a toxicological risk assessment.

Chapter 3

3.1. Final remarks

3.1.1. General discussion

3.1.2. Final conclusions/ Considerations

3.1. FINAL REMARKS

3.1.1. General discussion

In the last decades, aquatic pollution by pharmaceuticals has been recognized as a global environmental problem, jeopardizing the survival rates of many species. They have become predominantly detected in aquatic environments across North America and Europe leading to chronic effects on aquatic species. Thus, the understanding of the subjacent parameters of transcription levels of genes involved in neurotransmitter system is an uppermost need, as it can provide environmentalists with crucial information that may allow a sustainable future for the environment, because with a particular gene transcription level we can know whether the aquatic animal are to be chronically exposed to a PP**.**

In this study we confirm that *sert, 5-ht$_{3b}$, dopamine d$_3$, vmat* has higher degree of homology with *D.rerio*, *O.latipes, T.rubripes*, including *H. sapiens* and that in a phylogenetic tree based on the multiple alignment analysis in all sequences are well clustered within each corresponding group. Thus, it is possible that the aquatic specie selected for this study, the European seabass, can be exploited as a sentinel species for environmental field studies.

Expressional changes of neurotransmitters genes and receptors were determined upon FLUX and VEN exposure in European seabass. After the identification of the target partial sequences genes in this aquatic specie, we had to answer the question, whether PPs act differently on the neurotransmitters, by gene expression of dopamine and serotonin receptors, transporters specific and non-specific transporters and enzyme degrading measured by qPCR-RT.

The chronic exposures were performed for 21 days, followed by a 7 day depuration period, to assess the reversibility of effects. Different profiles of mRNA expression were obtained after exposure to SNRI and SSRI. Exposure to FLUX resulted in an acute response at day 1 in all analyzed target genes, except for the monoamine degrading enzyme, *mao*, which was increased after 21 days. Exposure to VEN caused significant differences at day 21 for the receptor of serotonin *5-ht$_{3a}$*. For both serotonin receptors lower mRNA level were detected, but only for *5-ht$_{3a}$* was significant different. Dopamine D_2 and D_3 receptors were significantly increased at 7 day of the recovery period.

Hence, is very important to consider the potential for environmental SSRIs and SNRIs, because they act in human and fish altering monoamines concentration as can see in this study. They can alter the neurotransmission due to changes in transcription levels of gene expression of serotonin and dopamine receptors. Activation of receptors on post-synaptic neurons opens ligand gated ion channels, which regulate the action

potential, and therefore, the signal transmission in neurons (Lodish et al., 2000). Due to the importance of neurotransmitters their increase understanding of the roles neurotransmitters plays in controlling fitness in fish.

3.1.2. Final conclusions/ Considerations

The present dissertation demonstrated the potential of two classes of antidepressants, SSRI and SNRI, to differently regulate gene expression in European Sea bass brain. Of particular interest, FLUX acute induction was regulated significantly in all target genes and response to VEN was observed as a chronic tendency in all genes, suggesting that they affect different biological pathways despite their shared pharmacological use of inhibiting the reuptake of serotonin by blocking serotonin or dopamine receptors.

These expression patterns indicate enrichment of human biological processes involving neurotransmission system. Thus, it can be made to biological processes associated with specific neurological disorders that have known or suspected environmental etiological components with others species or mammals. The elucidation of gene expression profiles for European Sea bass exposed to these SSRI and SNRI presents a unique comparison for other investigations on the effects of two classes of these antidepressants in fish and hypotheses for further research in fish physiology and the ecotoxicology of SSRIs or SNRIs present in aquatic environments.

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Appendixes

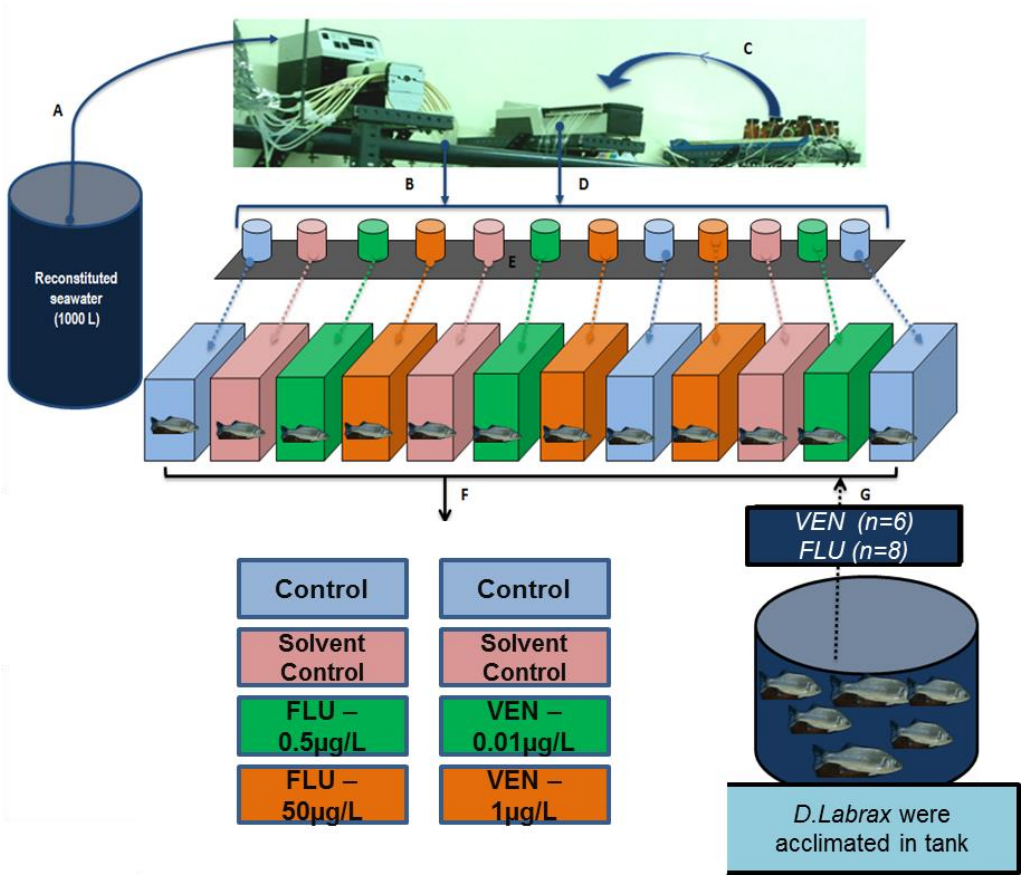
Appendix I

Samples types, country and concentrations reported of antidepressants

PP		Sample types	Country	Concentration reported (ng/L)	Year	Reference
FLUX		Portuguese effluents	Portugal	16,6-21,5	2014	Silva et al., 2014
		WWTP influent	Portugal	<17-3645	2011	Salgado et al, 2011
		WWTP influent	Portugal	<5	2011	Sousa et al., 2011
		WWTP effluent	Portugal	<4	2011	Sousa et al., 2011
		WWTP effluent	Spain	19-929	2007	Bueno et al., 2007
		WWTP influent	Spain	26	2009	Gros et al., 2009
		WWTP effluent	Spain	16	2009	Gros et al., 2009
		Hospital Effluent	Spain	4-100	2007	Gomez et al., 2007
		Ebro river	Spain	3	2009	Gros et al., 2009
		Jarama River	Spain	13-16	2010	Alonso et al., 2010
		Manzanares	Spain	22		Alonso et al., 2010
		River Guadarrama	Spain	13-120	2010	Alonso et al., 2010
		River Henares	Spain	11	2010	Alonso et al., 2010
		River Tajo	Spain	12	2010	Alonso et al., 2010
		WWTP efluent	Canada	50-99	2003	Metcalfe et al., 2003

		Peterborough, efluent	Canada	50±5	2008	Metcalfe et al., 2003
		Burlington STP efluent	Canada	38±3	2008	Metcalfe et al., 2003
		Hamilton Harbovir	Canada	13±1	2008	Metcalfe et al., 2003
		Hamilton/Harbor/Little River	Canada	13-26	2008	Lajeunesse et al., 2008
		WWTP efluent	Norway	0.6-8.4	2008	Vasskog et al., 2008
		Streamflow	Boulder Creek	9±6	2010	Schultz et al., 2010 (b)
		Streamflow	Fourmilw Creek	4.4±0.5	2010	Schultz et al., 2010 (b)
NorFLUX		WWTP influent	Portugal	<14-32.228	2011	Salgado et al., 2011
		WWTP influent	Portugal	45-105	2011	Sousa et al., 2011
		WWTP effluent	Portugal	60-240		Silva et al., 2014
		WWTP effluent	Spain	1141	2007	Bueno et al., 2007
		WWTP influent	Canada	11	2010	Metcalfe et al., 2010
VEN		Effluent	Texas	1310	2010	Schultz et al., 2010
		Streamflow	Boulder Creek	220±40	2010	Schultz et al., 2010
		Streamflow	Fourmile Creek	210±40	2010	Schultz et al., 2010

Appendix II



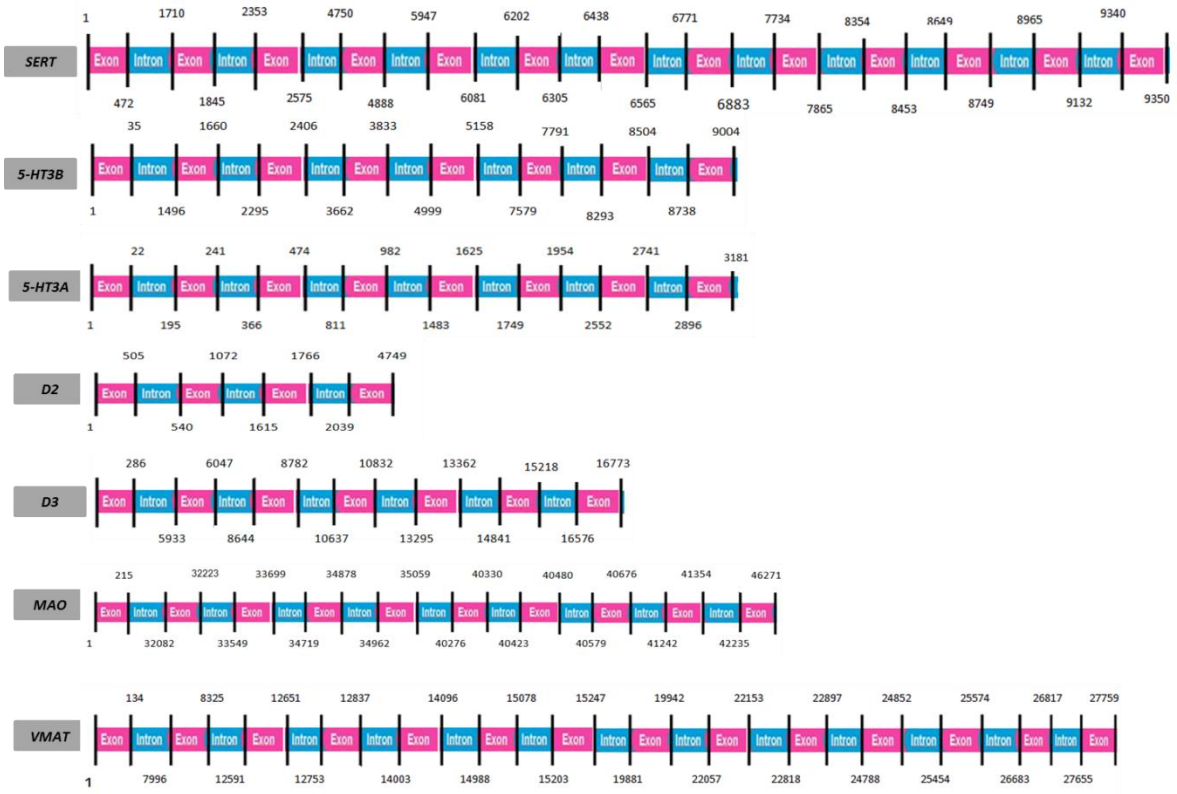
Exposure system of juvenile european Sea bass with FLUX and VEN.

The reconstituted seawater is conducted to a peristaltic pump (B) in which it forwards to a mixing container (E). C) From the stock solution of antidepressants with different concentrations are forwarded to another peristaltic pump (D) and forwards to the mixing vessels (E). E) From the mixture of bottles with the concentration of the contaminant and reconstituted seawater is continuously distributed to each of the respective tanks. After a day acclimatization of the animals in the experimental system of fluoxetine 24 are added to each of the tanks 12 (3 treatments with three replicas) with a total of 288 (G). In the case of venlafaxine 12 are added to each aquarium fish, with a total of 144 animals.

Appendix III

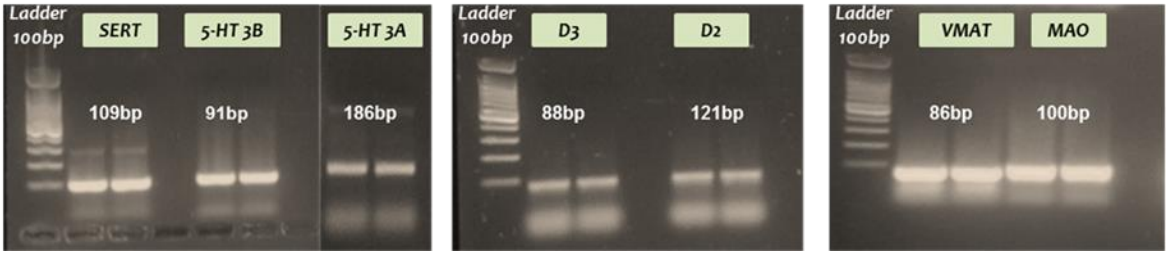
Genomic organization of the target sequences

In the present study our attempts to develop PCR primers based on published sequences. New sequences were defined from DNA available of the target sequences, as SERT, 5-HT_{3A}, 5-HT_{3B}, D_2, D_3, MAO and VMAT. The results defined all the intro and exons sizes of target sequences and the final length. From DNA was design primers removing the all the introns.



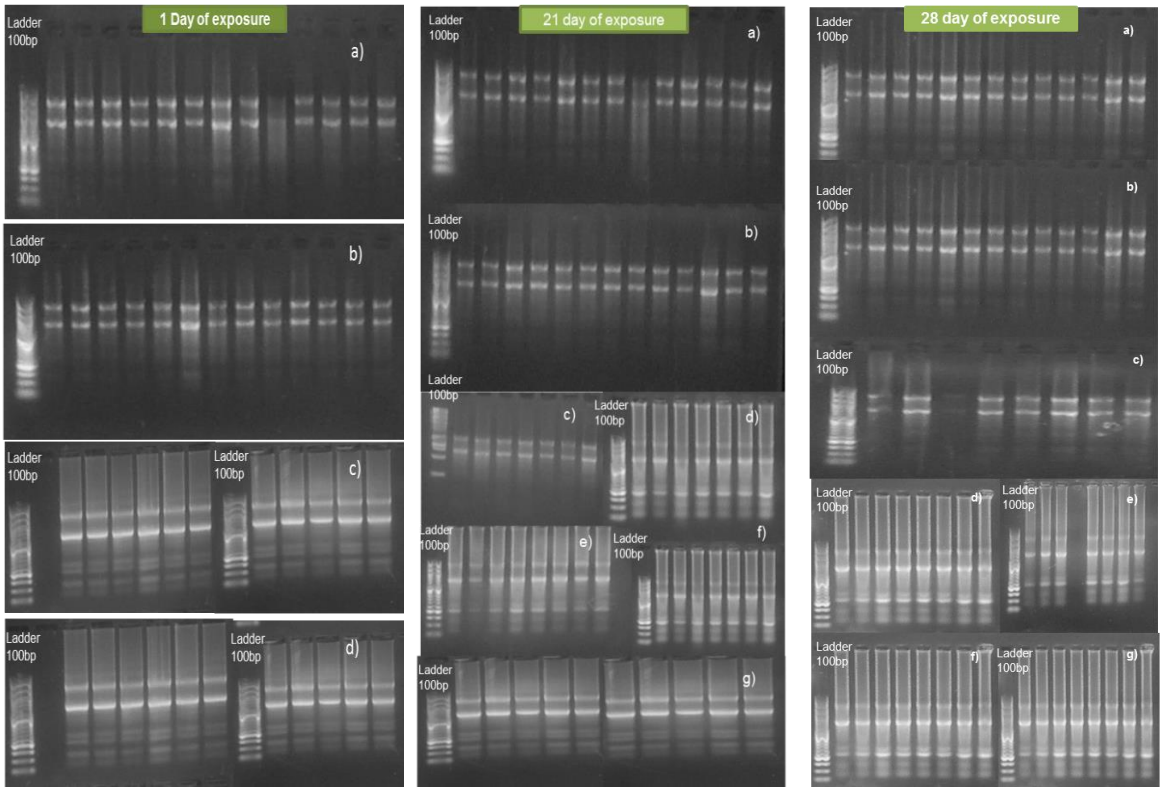
Schematic representation of the genomic organization (DNA) of target sequences. The exact sizes of the exons and the introns are given the top of each partition.

Appendix IV



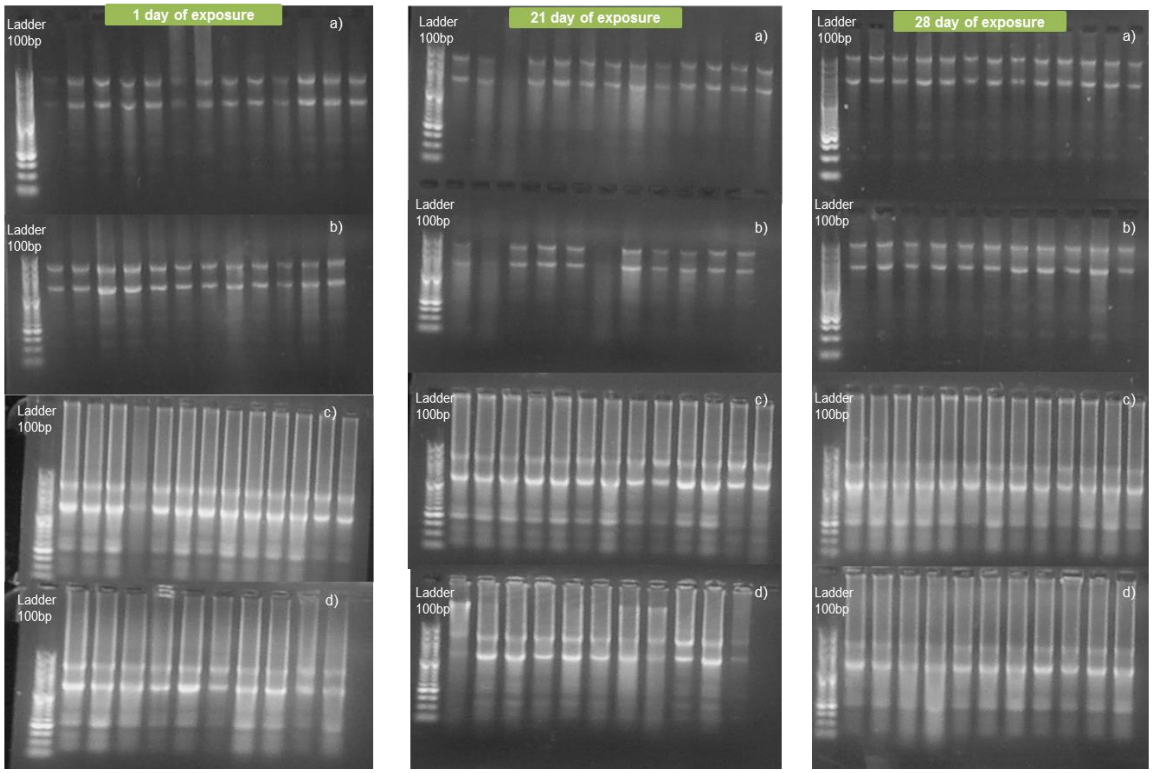
Electrophoresis analysis of PCR products amplified with selected primers in this study with 1.5% agarose gel.

Appendix V



Electrophoresis analysis (1.5% agarose gel) to verify the quality of the samples. Molecular weight marker (100bp ladder). 1 Day of exposure: a) and b) RNA samples after was subjected to digestion of DNA genomic; c) and d) Total RNA. 21 day of exposure: a), b) and c) RNA samples after was subjected to digestion of DNA genomic; d), e), f), g) Total RNA. 28 day of exposure: a), b) and c) RNA samples after was subjected to digestion of DNA genomic; d), e), f), g) Total RNA.

Appendix VI



Electrophoresis analysis (1.5% agarose gel) to verify the quality of the samples. Molecular weight marker (100bp ladder). a) and b) RNA samples after was subjected to digestion of DNA genomic; c) and d) Total RNA.